SOME STUDIES IN THE FORMATION

OF

ELASTIC TISSUE

Thesis presented for the degree

of

Doctor of Philosophy

by

Yusuf Y. Akrawi, M.B., Ch.B.(Baghdad).

Department of Pathology
University of Edinburgh.

August, 1947.

C O N T E N T S.

		Page.
Introduction. ...		i
Chapter I -	Specific Elastic Tissue Stains.	1 - 14
Chapter II -	The Development of Elastic Tissue in the Blood Vessels of the Chick Embryo.	15 - 33
Chapter III -	The Production of Elastic Tissue in Vitro.	34 - 72
Chapter IV -	The Development of Elastic Tissue in Canalising Blood Channels.	73 - 90
Appendix.		91 - 139
Chapter V -	Elastic Tissue Changes in Mammary Carcinoma.	140 - 166
Acknowledgments.		168
References. ...		169 - 180

---oOo---

INTRODUCTION.

This work started as a study of elastic tissue formation around canalising channels in occluded arteries. However, it was soon found that a general study of elastic tissue formation was necessary, since there were so many divergent opinions about it. The theories dealing with it were evolved around the beginning of this century and very few investigations have been made in recent years. On the other hand, the properties of, and changes occurring in elastic tissue, particularly in relation to vascular diseases, have been carefully studied (see Cowdry, 1933, Wilens, 1937, Saxton, 1942 and Hass 1939-1943). These investigations showed clearly that the physiological and functional needs of the body exerted an influence on the genesis and development of the elastic tissue. The extent of that influence, and whether the elastic formation was predetermined and inherent in certain cells, remained undecided.

The study of elastic tissue development in the chick embryo by histological means did not give definite answers to these problems, so it was decided to study it under experimental conditions in tissue culture. The correlation of all these findings led to the conclusion that the presence of certain special cells, which differentiate early in embryonic life, is necessary for that development. Such cells act on relatively simple chemicals present in the body, and elaborate elastic tissue, which is deposited in selective sites.

In/

In the adult, the elastic tissue appeared to develop along similar lines. The special elastic-producing cells present in the tissues can proliferate in response to certain physiological and pathological stimuli: this process was studied both in occluded arteries and in breast carcinomata.

The only criterion we have for detecting the elastic element in tissues at the present time, is the evidence adduced by the special elastic stains. Some time was spent on the study of their properties and mode of action, in order to select a standard stain: the results of this investigation are given in the first chapter on this work.

CHAPTER I.

SPECIFIC ELASTIC TISSUE STAINS.

SPECIFIC ELASTIC TISSUE STAINS.

The specific staining of elastic tissue played an important part in advancing our knowledge about the normal and pathological behaviour of this tissue, particularly in relation to vascular diseases. Most of the specific elastic stains were evolved about the beginning of this century. Before that, many non-specific stains were used, accounting for some of the confusion that existed as regards the histogenesis, and role of elastic tissue in disease.

Until better methods of determining the presence of the elastic element in tissues are discovered, its optical and tinctorial properties will remain as the only practical means of identifying it, and hence the importance of the specific stains.

It is intended here to discuss only briefly the merits of these specific elastic stains, without mentioning the details of their preparation and use; these can generally be found in the standard books on micro-technique (see References). The theory of their mode of action is not fully understood; this is due to our incomplete knowledge of the structure and chemical composition of the elastic tissue. It is known that the elastic element is acidic in reaction and has strong reducing powers.

Both/

Both properties are due to its high content of amino acids. An attempt will be made to explain the way each specific elastic stain acts according to these properties.

Samples of blood vessels taken from both the adult and foetus and skin from the mammary region were examined. They were taken from the same body for each series of experiments. The stains were tried also on elastic fibres isolated by digesting pieces of aortic tissue by the formic acid method (see Hass - d -).

Fixation:-

Fresh pieces of such tissues were fixed in different solutions. The effects of such fixatives on the elastic tissue was compared by staining sections of the fixed tissues with the three specific elastic stains.

The fixing mixtures tried were Zenker-Helly's, Bouin's, Carnoy's, Susa's and Formol-Corrosive Sublimate. The shrinkage of the elastic tissue was about equal in all of them. They all resulted in satisfactory elastic staining except after using Susa's fixative. It yielded inferior results when Weigerts elastic stain was used. The Zenker-Helly and Formol-Corrosive Sublimate fixatives were preferred as they helped in obtaining better nuclear and cytoplasmic staining as well. Formalin, especially when used as 10% formol saline proved to be the best of the simple fixatives tried.

Staining:/

Staining:-

Only the principles on which the old methods worked are mentioned here. It can easily be seen that the commonly used elastic stains are modifications that incorporated many of these principles.

1. The Maceration Methods.

These were the first to be used. By putting tissues in strong acids the collagen fibres swelled, leaving the unaffected yellow elastic fibres showing against a hazy background. Later (see 4, a.), various basic dyes were added to colour the elastic fibres while the swollen collagen fibres did not take the stain. Acids are now added to the specific stains for the same purpose. Alkalis were also used with the same results, but were given up since they made the collagen fibres quite unrecognisable (Krzysztalowicz).

2. The Impregnation Methods.

The Aniline dyes are deposited in tissues where metallic stains have been precipitated beforehand. By using Flemming's fluid as a fixative, the elastic tissue reduces the osmic acid in the solution and metallic osmium is deposited on the fibres, thus enabling them to stain selectively with any of the aniline dyes such as Dahlia, Victoria blue, etc. (Unna 1928).

3./

3. Methods using Acid Dyes.

a. Orcein: was the first dye to be used as a specific elastic stain. Taenzer (1889) was the first to use it. The present day formula is Unna's modification in 1891 (see Mallory) and will be more fully discussed.

b. Congo Red: Matsuura in 1924 used it as an elastic stain for frozen sections (see Conn). Krajian (1934) used it for the same purpose in a triple dye stain. I have tried the latter stain and it showed the bigger elastic fibres as golden yellow but the smaller fibrils were not easy to recognise.

4. Methods using Basic Dyes.

a. with an acid: vide supra.

b. with a mordant: this is the most important group since it contains both the Weigert and Verhoeff elastic stains.

The only important elastic stains that are now in common use are the Verhoeff, the Orcein and the Weigert elastic stains. These will be dealt with in more detail.

Verhoeff's Elastic Stain (Verhoeff 1908):

This Stain colours the elastic fibres blue-black. The rest of the section is left unstained. Many workers prefer it because of this and because of the sharp contrast it gives in photomicrography./

photomicrography. It was found to have the following disadvantages:-

1. The sections required careful differentiation to decolourise the rest of the tissues in the section without at the same time removing the stain from the elastic fibres.
2. The smaller and more delicate elastic fibrils are not usually seen in the final preparations as compared with the Weigert and Orcein Elastic stained ones.
3. The normal and degenerating elastic fibres were stained alike without discrimination.

The best way to overcome the first two disadvantages was to stop the differentiation with the ferric chloride solution, when most of the small elastic fibres and fibrils still retained the stain. By then, the rest of the section was not usually well cleared of the excess stain. The iodine was then removed by treatment with 95% Ethanol and the section was counterstained with Van Gieson's connective tissue stain (1 part of 1% acid fuchsin and 25 parts of a saturated aqueous solution of picric acid). The excess picric acid in the counterstain acted as a mordant and helped to remove the excess deposited stain from the background, without interfering with the staining of the small elastic fibres.

The Verhoeff stain is the only one in common use out of a large number of Haematoxylin stains that were used at different times/

times to stain the elastic fibres (see Mann, Thüringer, Romeis and Hatcher). All these stains are not specific in their action and may stain other tissues besides the elastic tissue. But the dye-lake formed by the combination of the basic dye Haematin, and the mordant ferric chloride, possesses strong basic properties and so tends to combine more firmly with the acid-reacting elastin, than with other tissues in the section. Next come muscle and cellular cytoplasm. The collagen is the tissue that tends to retain it least. In the differentiation process, the ferric chloride solution acts as a mordant differentiator and tends to dissolve the Haematin out of the precipitated dye-lake, and so clears the stain out of the tissues. Since the rest of the tissues adhere to the stain less firmly than elastic tissue they give up the stain first. If the differentiation is allowed to continue long enough, the elastic fibres will also begin to be decolourised. This is actually what happens if we try to remove the stain completely from the background. But if it is stopped before that, then the excess picric acid (which is an acid differentiator) will remove that stain from the background by substituting itself in the tissues that have more affinity for it than for the iron Haematoxylin (muscle, cellular cytoplasm, etc.)

Verhoeff's elastic stain proved useful for routine staining but gave inaccurate and inconsistent results where the delicate elastic fibres were meant to be seen. The differentiation was also very tedious when serial sections were being stained at the same time.

The/

The Taenzer-Unna Orcein Stain (Mallory 1938):-

This acid dye stained the elastic fibres selectively and gave consistent results. Many modifications of this stain have been introduced for the purpose of either shortening the staining time or to prolong the life of the staining solution since it was found that it lost its potency with keeping. Such changes generally either altered the proportions of the water and alcohol in the stain or substituted some other acid for the HCl (see Schmorl). In these experiments the best results were obtained by using Unna's formula and staining the sections for 24 hours at room temperature. The solution was prepared by using Grübler's Orcein which kept its potency for 1½ years. When other Orcein preparations were used the solutions did not keep for more than 2 - 3 months. It appears that the quality of the orcein, which is extracted from certain fungi, is the determining factor. Since the beginning of the last war synthetic Orcein has come into use, and is highly recommended by both Salthouse (1945) and Lendrum (1946). The former thinks that it is even superior in its results to the natural one. I have had no opportunity of using it.

Orcein is a selective elastic stain, simple to use and control. It colours the elastic tissue deep reddish-brown and the rest of the tissues in the section light brown. If the section is washed in 1% HCl in water, after the differentiation in acid alcohol,/

alcohol, then the background staining is removed. It was found best not to use a counterstain after orcein since these tended to change the colouration of the elastic fibres. Only the nuclei were stained by haemalum. This had the additional advantage of showing clearly the calcium deposits present in sclerosed vessels, a thing which the Weigert elastic stain did not show so distinctly.

Orcein showed the small elastic fibrils with constant accuracy only equalled by the Weigert stain. It can also differentiate between the normal elastic element (elastin) and the degenerating kind (elacin). It was not used in this work more frequently, because of the difficulty in using counterstains after it which were indispensable. Its use was mainly as an additional confirmation of the presence of elastic fibres that were shown by the Weigert stain.

The stain works by having the acid orcein deposited on the elastic fibres, through its reduction to an insoluble form by the elastin. The Hydrochloric acid is added to the solution to keep it more stable by preventing its reduction on keeping, and it also helps to hydrolyse and swell the collagen fibres, making them refractory to the stain.

Weigert's Elastic Stain (Schmorl 1914).

This stain is universally recognised as the best and most specific of the elastic stains. Few other stains have had so many/

many modifications and so many articles written about them (see Krzysztalowicz, Mann, Langeron, Unna, Bujard, Cretin and Mattot, Hatcher, Haynes, Lawson, Lendrum, Romeis, Wollart and Houette, and Schmorl). Most of them agreed that it was the best elastic stain and also tried to deal with these two disadvantages that the stain possesses:-

1. The background stains a deep violet colour which is difficult to remove by differentiation.
2. The staining solution loses its potency with keeping.

Many have attributed the cause of the deterioration to the amount of ferric chloride in the stain and have either increased or decreased it. Some blamed the amount of Hydrochloric acid and so increased it or substituted it for another. Others added other chemicals such as acetone or phenol to prolong the life of the solution.

Other modifications varied the concentrations of the different dyes and reagents in the stain, substituting them for others of the same groups, or varied the methods of preparing the stain. All this was done to prevent the background staining. Trials with most of them, showed that the best two modifications were those done by Hart (Mallory) and French (Lendrum).

The original Weigert formula gave an excellent staining solution if it was prepared in the following way:-

2 gms. Basic fuchsin and 4 gms. Resorcin are boiled in 200 ccs. of distilled water for 15 minutes, stirring all the time. 25 ccs. of 30% ferric chloride solution are added gradually and the mixture boiled for another 5 minutes. It is then cooled and filtered. The precipitate on the filter paper is washed with distilled water until no more colour appears in the filtrate. The precipitate is then dried, dissolved in 200 ccs. of Ethanol, boiled for 5 minutes, cooled, filtered and 4 ccs. of Hcl are added to it. By carefully washing the precipitate on the filter paper the excess ferric chloride and uncombined basic fuchsin were removed. The latter especially appeared to be the part responsible for the background staining. The sections were kept in such a staining solution for $1\frac{1}{2}$ - 2 hours. After that, all that was necessary was to wash the section in ordinary Ethanol and proceed with the counterstaining. Sections kept in this stain even for 6 - 8 hours required no other treatment and showed no traces of background staining. Another advantage was, that by preparing the staining solution in this way, its potency did not deteriorate with keeping. A solution prepared 14 months ago is still giving good results.

The Weigert stain colours the elastic fibres violet-black. It does not require any careful control or differentiation and so was very convenient for staining many sections at the same time. Different counterstains such as Masson's trichrome stain, Van Gieson's stain, Neutral red, Phloxine, Lithium carmine, etc., can be used after it, without having any detrimental effects on the quality of the elastic staining. It was found convenient

to/

to stain the nuclei by Heidenhain's iron Haematoxylin. This was followed by Van Gieson's stain (1 : 20). The sections must be thoroughly washed with water, before using the latter stain, in order to remove all traces of acids. The collagen would otherwise stain deep purple instead of the usual bright red.

Another advantage of the Weigert stain was that it differentiated between the normal and degenerating elastic fibres in blood vessels. In cases where reduplication of the internal elastic lamina occurred, the inner layers stained normally, while the outer ones were refractory or only faintly stained. In later stages the degenerating elastic fibres began to undergo a series of colour changes, ranging from violet to red-brown, after Van Gieson's counterstain. This was especially noticeable in the swollen elastic fibres that were undergoing the so called Vitreous (Goodall 1908) or hyaline degeneration (Aschoff 1924). Hass (a) was of the opinion that the depth of the elastic colouration increased with age, and he attached no important significance to the tinctorial changes of the elastic fibres in disease. It is true that some broken elastic fibres stained like the normal ones, but it was found also that these colour changes signified loss of function, and could be safely regarded as signs of degeneration. The orcein staining of such fibres was lighter compared to the normal ones, while the Verhoeff stain stained all fibres alike.

The Weigert elastic stain is made up of a basic dye (Basic Fuchsin), ~~and~~ a diphenol (Resorcin) and a mordant of a tri-valent metal (Ferric chloride). Any other member of each of the three groups/

groups can be substituted in the formula, as was done in the various modifications advocated. The original formula is preferred because the dye-lake formed by the combination of basic fuchsin and ferric chloride has stronger basic properties and is more stable than any other combination of the two groups. Resorcin is a better reducing agent than the other diphenols. Hydrochloric acid helps to prolong the life of the solution by preventing the ferric salt in the dye-lake from changing to the ferrous state, and thus rendering the stain useless. The acid also swells the collagen in the section by hydrolysing it, and so renders it refractory to the stain. Alcohol is used to dissolve the stain since the dye-lake is insoluble in water. The staining process can be explained as a reaction between the highly basic dye-lake that has strong oxidising properties and the acidic elastin with the strong reducing properties. As a result of that reaction the dye-lake is precipitated permanently in the elastin only. When the sections are washed in ethanol (or acid alcohol) all the stain loosely linked with the other tissues, will redissolve, leaving the elastic fibres staining violet-black against a colourless background. Resorcin acts as a further reducing agent in the reaction.

Conclusions:-

At the present time the physical and optical properties of the elastic tissue, do not form a practical method of determining its presence in tissues (Wilens, Hass d and e). The chemical analysis/

analysis of the elastic tissue is very complicated and no definite results were obtained, since it was difficult to isolate it in a pure form (Tritchkowitch, Jordan, Stein, Lowry and Hass d). The new methods of fibre X-ray photography have so far given no accurate means of identifying the elastic fibres (Astbury). So we still have to rely mainly on the shape and tinctorial properties of the elastic tissue to determine its presence. This point is stressed here, since it forms the only practical way of determining the nature of the first elastic fibres to appear in the organism or in culture. Many workers have described a stage in the development of the elastic fibres when they do not stain specifically. This theory has not been proved conclusively and we still feel that our best guide to the first appearance of elastic fibres in tissues is the elastic stains.

The experiments done on staining lead to the conclusion that the Weigert elastic stain is the most specific and consistent of all. In the following chapters it will be used as an indication of the appearance of early elastic elements in tissues.

CHAPTER II.

THE DEVELOPMENT OF ELASTIC TISSUE IN THE BLOOD VESSELS OF THE CHICK EMBRYO.

THE DEVELOPMENT OF ELASTIC TISSUE IN THE BLOOD VESSELS OF THE CHICK EMBRYO.

Although many of the early anatomists must have noticed the elasticity of various tissues, it was left to Henle, the Father of Histology, to make the first comprehensive studies on elastic tissue and to describe such a special tissue in the walls of blood vessels in 1841 (Hass - a -). Since then, several workers have attempted to explain how the elastic tissue develops in both the embryo and the adult. Most of the evidence was based on histological techniques, until recently when tissue culture methods were employed. The suggested theories differed principally, on whether the elastic fibres were formed intra- or extra-cellularly. These theories changed with the changes in the conceptions regarding the formation of the embryonic mesenchyme, and the development of the fibrillar ground substances. Many of the conflicting results could be accounted for, when we know that many of the investigators used non-specific stains to demonstrate the elastic fibres.

I. The Intra-Cullular Theory:-

Each part of the connective tissue cell was at one time or another thought to be responsible for the formation of the elastic fibres:

a. Henle, Heller, Retterer suggested that the nucleus was the responsible part (see Röthig).

b./

b. Others, thought that the elastic element developed first in the form of granules in the cytoplasm, became arranged in pearl-string fashion in the outer half of the cytoplasm and then fused together to form elastic fibrils. These separated from the cells as such (see Jores, Fischer).

c. Some workers agreed that the elastic fibrils developed directly as such in the cytoplasm (Loisel, Fuss).

d. Many of the supporters of the intra-cellular theory believed that they developed by the transformation of the cellular cytoplasmic prolongations, into elastic fibrils. These surrounded the cells in what were termed "Elastic-mantles" (Nakai, Geipel, Ladwig, Krompecher).

e. Flemming and Hansen (see Mall) divided the cytoplasm of the connective tissue cell into exoplasm and endoplasm. The former formed collagen and the latter formed elastic fibres.

f. More recently, De Kervily (1924), Orsós (1926) and Odiette (1934) recognised pre-elastic argyrophil granules or fibrils. These developed in the cytoplasm, changed into elastic fibrils and then separated from the cells.

Some investigators did not agree that the general connective tissue cell, or fibroblast, formed both the collagen and elastic fibres. Loisel recognised, early in the embryological development, certain mesenchymal cells developing into "elastoblasts." These were, according to him, the only cells capable of forming elastic tissue in the embryo but not in the adult. Krompecher

(1928)/

(1928) concluded that the same occurred in the adult tissues. Odiette came to a similar conclusion with tissue culture.

II. The Extra-Cellular Theory:-

The early supporters of this theory believed that elastic fibres, like collagen, formed as part of the lifeless ground-substance with the cells taking no part in the process.. Their best argument was that they never saw any intra-cellular elastic granules or fibrils (see Röthig, Hass - a -). This theory of fibre formation gained much support when Baitsell (1915) showed that fibres can be formed by the organisation of plasma, without cellular participation. Wolfe (1931) reached similar conclusions regarding elastic tissue. This theory suggests that both collagen and elastic fibres are formed, inside large cell-free spaces, by the gelation of certain body sols, independent of cellular activity.

III. Origin from Collagen.

Some investigators, finding no specially-shaped cells to account for the elastic fibre formation, especially in the adult, suggested that they were formed by the transformation of collagen into elastic fibres (see Loisel, Röthig). The recent supporters of this theory (Bierich, 1922, Buerger, 1924) explain the process as due to an alteration in the physico-chemical composition of the collagen brought about by physiological or pathological processes occurring in the organism.

IV/

IV. The Combined Theory:-

This is based on Mall's Syncytial Theory. It supposed that early in embryonic life, the cells of the undifferentiated mesenchyme were connected together into a syncytium by protoplasmic processes. The peripheral parts of the common cytoplasm form the different ground substance materials, while the inner parts surround the nuclei and form the different connective tissue cells. Accordingly, both the connective tissue cells and ground substances, which till then were separated, are of one origin. The elastic fibres are formed in the outer framework of the common cytoplasm (Mall 1902) and it is a matter of individual opinion whether one considers them as part of the cells or ground substance (Geipel, 1906).

A recent elaboration of this theory of fibre formation is widely accepted. Ranke suggested that at first, undifferentiated argyrophil fibrils or "Gitterfasern" are laid down. These, under chemical or mechanical forces, depending on the developmental requirements of the organism, change into either collagen or elastic fibres. (Kraupse, 1922, Hueck, 1922, Day, 1936). Tissue culture experiments on the formation of collagen fibres favour this conception (see next chapter). The only differences amongst the supporters of this theory as far as it applies to the formation of the elastic fibres are whether -

1./

1. The pre-elastic argyrophil fibres and the reticulin fibres are one and the same (Kraupse).
2. These fibrils are impregnated with the elastic element which is secreted by the cells (Hueck, Jalowy, 1937).
3. The argyrophil fibres by themselves change chemically into elastic fibres under the physiological influences and demands of the body (Bujard, 1935).

The effects of the physiological demands upon the formation of elastic tissue have been stressed by several authors (vide supra). Although many tissues in the body possess "elastic" properties (in the physical sense of the term), recent work has shown that elastic fibres form the best tissue for the dissemination of stresses directed at isolated points, for the co-ordination of rhythmic movements of separate units, for the maintenance of the tone of an organ and for the prevention of injury from any excessive force (Hass - a -). It is the tissue that would yield best to a stretching force but would regain its original shape at once when the tension is released. The best illustration of how these elastic properties are utilised in the body is found in the way in which it is distributed in the vascular system. Thus it is concentrated in the great vessels where it helps to keep a continuous flow of blood during diastole, and decreases in the smaller vessels gradually till arterioles of 62 micros diameter are reached when all elastic/usually disappears from the wall (Maximow & Bloom). (See also Bramwell, element

Bramwell/

Bramwell et al, Foulton and Mcswiney for the physiological functions of elastic tissue). How these physiological functions influence the formation of elastic tissue, whether the effects are chemical or physical in nature and in either case whether they are directly responsible for the formation of elastic fibres without inherent cellular activities is debatable.

The following experiments were made in order to draw our own conclusions to many of the questions, related to the formation of elastic fibres, on which there are no general agreement.

Materials and Methods:-

It is generally agreed that the vascular system is the earliest to acquire elastic tissue during the embryonic development. The chick embryo was selected for that study because of the relative ease of determining its age accurately and because it was used in the tissue culture experiments for the same purpose (see the next chapter). Four to twelve day old embryos were taken out of their shells and immediately fixed in either Zenker-Helly or 10% formol saline. They were embedded in paraffin and serially sectioned at a thickness of 5 microns. Alternate sections were stained by the Weigert elastic stain and the Gordon and Sweets silver impregnation method (Lendrum 1946). At least four embryos were taken for each day period.

Findings:-

Rhythmic/

Findings:-

Rhythmic cardiac pulsations in the chick embryo started in the third day of incubation, coinciding with the completion of the vitelline circulation (Patten, 1925). By the fourth day the conus arteriosus began to form and about the fifth day the horizontal septum of the conus began to divide it. At the same time the sixth arch began to show signs of developing into the pulmonary artery (see also Kerr). None of the sections of the four or five days old embryos showed any traces of elastic tissue. In the sixth day of incubation, the horizontal septum had reached the lower end of the conus and now the aorta and pulmonary trunks could be recognised. Their walls were found to be made of a network of undifferentiated mesenchymal cells joined together by branching protoplasmic processes (fig. 1). Each cell had a large nucleus surrounded by a minimal amount of cytoplasm tapering at each end into the processes. The lumina of both vessels were lined by a layer of cuboidal cells. The walls of the aorta were thicker and the cellular structure more compact than that of the pulmonary trunk.

The first elastic element to be seen in the chick appeared in six-day-old embryos in the first part of the aorta. It was in the form of small granules which took the Weigert elastic stain and were situated in the spaces between the connective tissue cells (fig. 2). Such elastic granules were in no particular relation to each/

each other, but in areas where the mesenchymal cells were more compactly arranged, such granules were crowded together in the form of small fibrils (fig. 3). This appeared to be the process by which the elastic fibrils formed and was more obvious in 7-day embryos (fig. 4). Here the cells forming the wall of the aorta were packed closely together in the form of regularly arranged rows while the elastic granules and fibrils formed a sort of inter-cellular cement. In the septal wall, the cells were still less regularly arranged and so were the elastic granules and fibrils present amongst them, although these elastic elements were as well developed as in the aortic wall proper (fig. 5). At this stage the pulmonary artery began to acquire elastic granules and fibrils. Thereafter, the lines of development of that tissue in the pulmonary artery were similar to those seen in the aorta but the amount of elastic tissue developed was less (see fig. 7).

By the 8th day the septal wall had gradually changed into the adjoining walls of both the aortic and pulmonary vessels which were now completely separate from each other (fig. 6). The aorta had about 12 - 15 concentric layers of elastic tissue, separated from each other by a layer of cells. The outer elastic layers were better formed than the inner ones.

The development after that continued on similar lines and by the 12th day of incubation more new layers of elastic fibres were added/

added while the older ones grew thicker (fig. 7). Any further development till the hatching of the embryo was similar. The elastic tissue in the rest of the vascular system appeared gradually at later dates, starting with the big vessels (8th day) and spreading into the smaller ones.

As the number of elastic fibres in the walls of the aorta increased gradually day by day, the number of the connective tissue cells followed suit, but in a less conspicuous and obvious manner.

Silver impregnated sections of five-day-old embryos showed the development of argyrophil granules and fibrils amongst the mesenchymal cells forming the walls and septum of the conus. The six-day-old embryos had argyrophil fibrils and granules in exactly the same places in which the elastic granules and fibrils were found. As the elastic fibres became better developed so did the silver-stained fibres occupying the same position. This was always the case when alternate sections stained differently were examined. In order to confirm this, a double staining method of the same section was tried next.

The sections were first impregnated with silver and were then stained by Weigert's elastic stain or orcein. Such sections showed that the same fibres in the wall of the aorta were taking both the elastic and silver staining. The reticular fibres in the rest of the tissues were impregnated by the silver only.

Every/

Every fibre that took the Weigert stain showed argyrophilia as well (fig. 8). This was shown in all the sections stained with the two stains and taken from 6 - 12 day-old embryos. Later on the elastic fibres gradually lost their ability to take the silver stain so markedly, while the reticular fibres in the other tissue continued to show increased depth of impregnation with silver. It appeared that this argyrophilia of the elastic fibres was inversely proportional to the age of the fibres in the embryo.

Discussion:-

The first appearance of the elastic element in the chick embryo was in the form of extra-cellular granules in the walls of the first part of the aorta. These granules were always found in immediate proximity to the mesenchymal cells of the wall. No elastic granules or fibres were seen to develop in areas where the cells were absent. The first granules were scattered over a large area in the spaces between the cells. As the cells became more crowded so did the granules which were now beginning to surround each one as with an elastic mantle. The re-arrangement of the cells into more compact and uniform layers helped the granules to fuse together and form elastic fibrils. This process can be easily followed histologically in these preparations. The further growth of these fibrils into fibres appeared to be through the deposition of more elastic element, directly or in the form of granules, on them. No evidence of side-to-side fusion of fibrils to form thicker ones was seen as suggested by many (Nakai, Geipel,/

Geipel, Röthig). The growth in the length of the fibres occurred through the link with new elastic granules and fibrils forming in the more distal parts. The formation of the elastic fibres in the vascular system was like that of a chain, first beginning in the aorta and gradually spreading into the great vessels and more peripheral parts as the embryo became older.

It has already been mentioned that the first elastic elements developed extra-cellularly but in the neighbourhood of cells. We have seen how the cells help mechanically in forming the fibres but whether they are responsible for producing the elastic element or not cannot be conclusively proved here. The first appearance of the elastic element coincided with the start of function in the internal vessels of the embryo and it could always be argued that the development of the elastic tissue was due to the physiological demands brought about by the start of function, and not due to an inherent activity of the mesenchymal cells in the vessel walls. The cells, in the proximity of which the elastic tissue was forming, had no special morphological characteristics to distinguish them from other mesenchymal cells, and hence all the doubt about their role in elastic tissue formation. This however does not exclude the presence of such cells. J. Huxley (1924 and 1932) suggested that when the cells of the three embryonic layers develop into the various tissue cells, they first pass through a stage of chemo-differentiation, when the cells are still morphologically similar in appearance but have different biochemical/

biochemical actions depending on the type of tissue they are destined to become. After that, most of the cells become histologically differentiated as well. Many of the mesenchymal cells do not pass through the latter stage and remain morphologically similar to each other. The only way to distinguish between these cell types is by the different ground substance products they lay down (bone, collagen fibres, elastic fibres, etc.). Accordingly we can assume that some of the mesenchymal cells in the walls of vessels are especially responsible for the formation of elastic fibres although they show no morphological characters distinct from other mesenchymal cells, except that they are surrounded by the particular ground substance they produce. It is only in this sense that the term elastoblasts can be applied to them. This will be further discussed in the next chapter.

Whether such cells secrete the elastic element as such or only special substances or enzymes which act on the gels surrounding the cells, changing them into the elastic element, cannot be ascertained although the latter is the more likely to occur if we assume that the elastic fibres follow similar lines of development as the collagen fibres (Stearns, 1940).

The relation between the argyrophil and elastic fibres is difficult to place. It must be pointed out that, given enough time in the silver solution, any type of tissue in the section could be made to show argyrophilia. As far as possible all the sections/

sections in these experiments were stained by the same method, using freshly prepared solutions only and subjecting the sections to the same time factors. All the elastic elements in the sections of 6 - 12 day-old embryos showed argyrophilia. This can be interpreted in the following ways:-

1. That it is an atypical process of staining. It has already been mentioned that as far as possible the steps used were standardised and all the results obtained were the same.
2. The argyrophil granules and fibrils are the first stage in the formation of the elastic fibres which only later acquire the property of staining with the specific elastic stains. This can only be proved if the argyrophil granules and fibres are shown to possess the chemical properties specific for the elastic element. Since this is not possible yet we still have to rely on the specific elastic stains to indicate for us the first appearance of such a tissue.
3. The elastic fibres in the embryo show argyrophil properties being more evident in the earlier stages of their development. This will be discussed further in the next chapter.

From the above discussion it can be seen how the old histological methods are not satisfactory in helping to elucidate some of the problems of normal growth and differentiation and to give a clear conception of the processes involved in the normal development of the animal organism. This is why conclusions are not/

not drawn here, but will be left to the end of the next chapter after a close study of the development of elastic tissue by tissue-culture methods has been discussed.

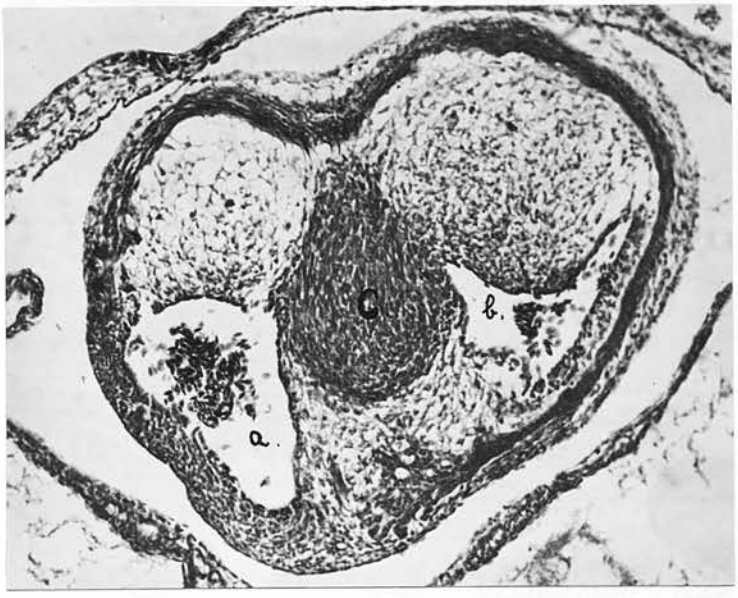

Fig. 1.

Transverse section of the conus arteriosus near its junction with the heart in a 6-day old embryo.
a. Aorta. b. Pulmonary artery. c. Horizontal septum.
Weigert - haematoxylin - Van Gieson x 140.

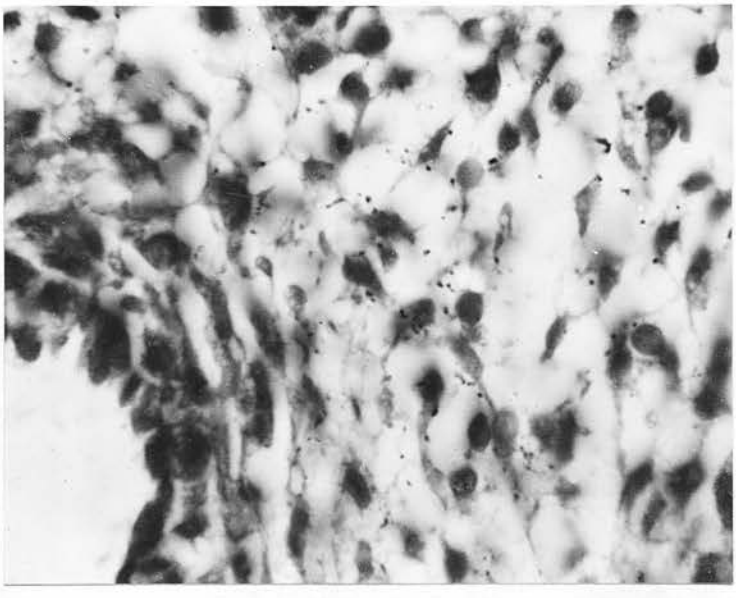

Fig. 2.

High-power view of Fig. 1.a. showing the appearance of the first elastic granules developing extra cellularly in the aortic wall.
Weigert - haematoxylin - Van Gieson. x.1000.

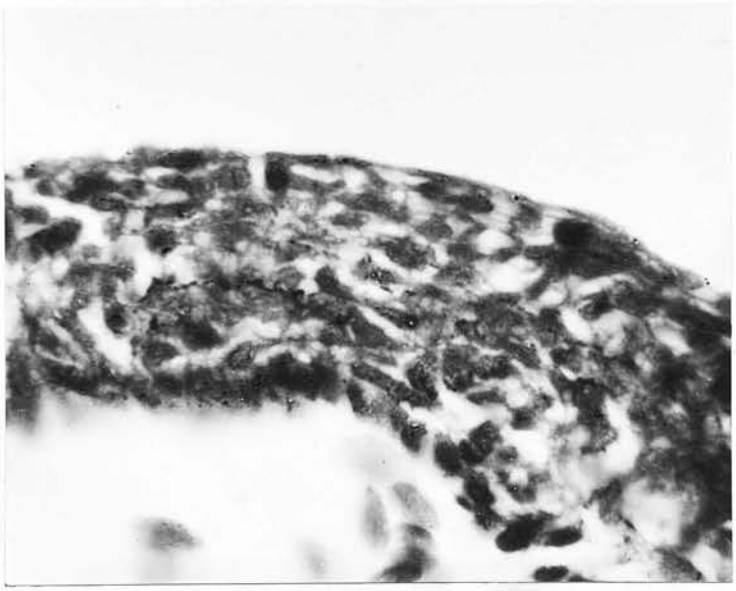

Fig. 3.

High-power view of Fig. 1.a. showing the aggregation of the elastic granules into fibrils in the more compact part of the aortic wall.
Weigert - haematoxylin - Van Gieson. x 1000.

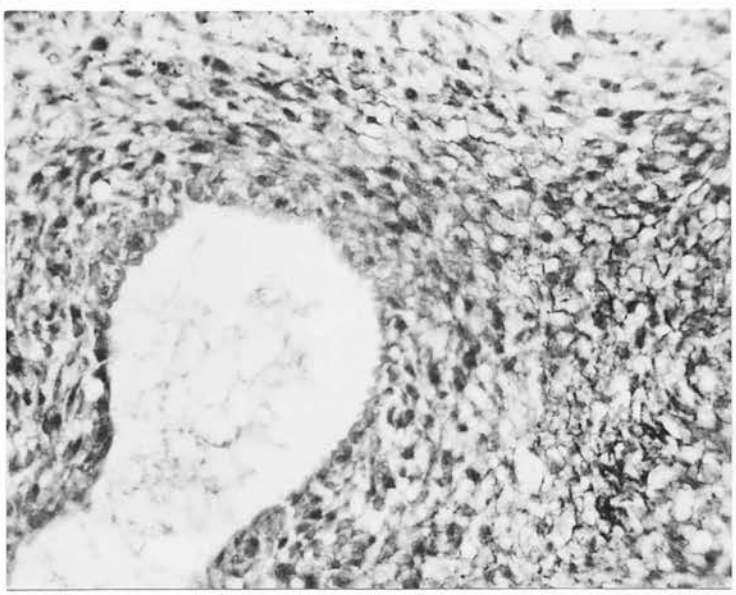

Fig. 4.

Transverse section in the aortic region of a 7-day old embryo. The small elastic fibrils are by now better developed.
Weigert - haematoxylin - Van Gieson. x 400.

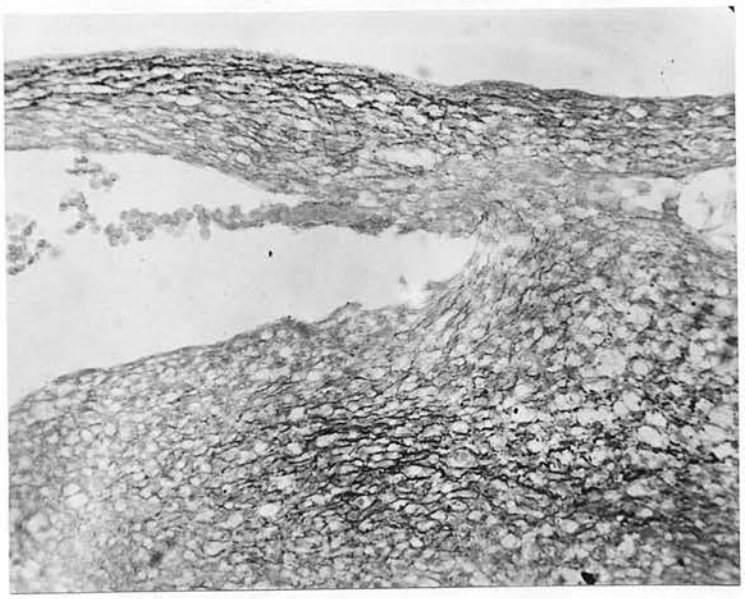

Fig. 5.

Longitudinal section in the aortic region of a 7-day old embryo. Note the granularity of the elastic network in the septal wall where the cells are not regularly arranged yet.

Weigert's elastic stain. x 400.

Fig. 6.

Transverse section of the aorta in an 8-day old embryo. The aorta has by now several regularly arranged elastic layers. The pulmonary artery has separated.

Weigert - haematoxylin - Van Gieson. x 140.

Fig. 7.

Oblique section through the aorta of a 12-day embryo, showing the elastic network at this stage. In - a - and - b - the elastic fibrils are still young and faintly stained.

Weigert - Van Gieson. x 90.

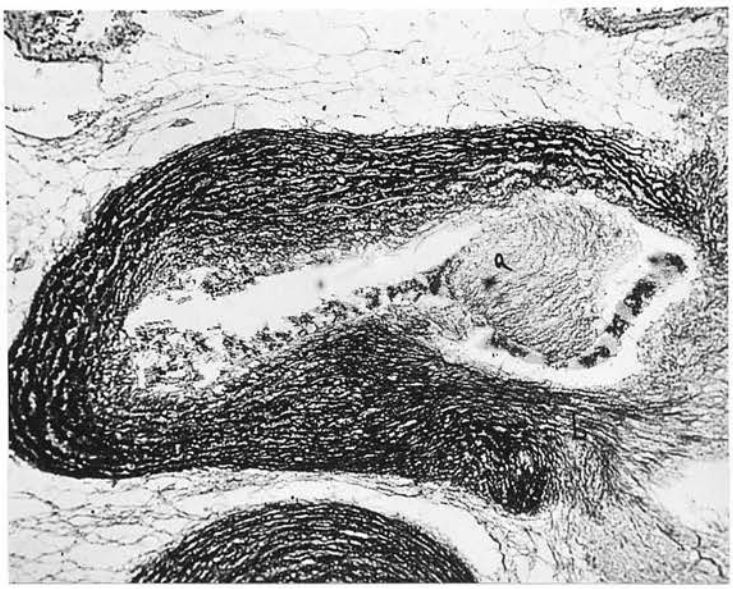

Fig. 8.

The next section (5 microns) to that shown in Fig. 7. The same fibres take both the silver and elastic stains. The small fibrils in - a - and - b - are well shown by the double stains.

Gordon & Sweete - Weigert stains. x 90.

CHAPTER III.

THE PRODUCTION OF ELASTIC TISSUE "IN VITRO".

THE PRODUCTION OF ELASTIC TISSUE "IN VITRO."

The process of elastic tissue formation in the embryo revealed how inadequate were the simple histological methods to explain that process. The purely morphological concepts of cells and tissues were not capable of explaining the building-up of elastic tissue in the embryo and so it was hoped that by using tissue culture methods, a better conception of that formation would be obtained. It is not intended to discuss the growth and metabolism of cultured cells and their reaction to the different media etc., but only to show the possibility of obtaining a new growth of elastic tissue and to study its formation and behaviour in vitro. It was thought that by observing the formation of elastic tissue under controlled experimental conditions and removed from the ordinary physiological influences that may affect them in the living organism, it would be possible to answer some of the questions connected with its formation.

The formation of connective tissue fibres in tissue culture was shown by Lewis (1917) to occur intra-cellularly. Baitsell (1915 and 1921) believed that the cells played no part in that formation and that the fibres were formed from the fibrin net of the plasma present in the culture medium. Maximow (1928), McKinney (1929) and Doljanski and Roulet (1933) all proved that the fibres could be formed in culture-media free of the fibrin network. They all agreed that the fibrils were formed extra-cellularly/

extra-cellularly but that the presence of cells was essential. In the case of the collagen fibres, they found that an extra-cellular argyrophil fibre was the first to form and which only later took the collagen stains.

The study of elastic fibre formation in vitro has not been as thorough as that of collagen fibres and the few results obtained were conflicting. Bloom (1929) obtained the fibres by culturing for ten days cardiac and aortic tissues of guinea-pig embryos. He found them forming extra-cellularly and thought that they formed a different network from the argyrophil fibrils. Porta (1930), using the same tissues from chick embryos, saw the new elastic fibres extra-cellularly after six days of incubation. Odiette (1933), also using chick embryo tissues, differentiated certain cells, the elastoblasts, in whose cytoplasm the first elastic fibrils developed after only twelve hours of incubation.

Materials and Methods:

Owing to difficulties of obtaining tissue culture equipment and to limited working space, only the simplest instruments and methods necessary for these experiments were used. This led to the limitation of the work, but on the whole the results obtained served the purpose. Maximow's double-coverslip hanging drop technique was used (Maximow, 1925, Cameron, 1935). The cleansing and sterilisation of the necessary instruments was done according to the instructions given by Strangeways, 1922, Cameron, 1935, and Parker,/

Parker, 1938, in their books on culture techniques, choosing whichever methods were found suitable. Tyrode solution (pH 7.4-7.8) was used as a physiological saline. The embryonic extract was prepared by mixing minced 8-10 day-old chick embryos with an equal volume of Tyrode solution. The mixture was centrifuged and the supernatant fluid taken and kept till use in a refrigerator. The plasma was obtained from a young adult cock by using the cardiac puncture technique. The blood obtained was mixed with Heparin to prevent clotting (1 c.c. of 1:5000 Heparin solution added to 20 c.c.s. of blood). It was then centrifuged and the plasma separated.

Tissues from the chick embryo aorta and large vessels were the ones mainly used in this work. Different ages were tried and the best growth of elastic tissue was obtained when using nine-to-twelve-day-old embryos. Such embryos were taken out of their shells, with aseptic precautions, and the aorta and big vessels were dissected out and put in special dishes containing Tyrode solution. They were then cut into small pieces each about 1 m.m. in diameter. The use of a dissecting microscope and sharp knives helped a great deal in avoiding undue tearing of the tissues which would be detrimental to their growth in culture.

By the aid of Pasteur pipettes a drop of plasma and a smaller one of embryonic extract were mixed on a No. I coverslip ($\frac{7}{8}$" diameter) which was attached to a thin, square glass slide (1.5" x 1.5"). A piece of cut tissue was then embedded in the centre/

centre of the medium, a depression slide was placed over the round coverslip and the preparation was sealed airtight by a warm paraffin-vaseline mixture (1 : 7). After the plasma medium had clotted the slides were turned upside down, thus making hanging-drop preparations. These were incubated at 37°C.

In order to give the fibres an opportunity to develop and modify themselves, no sub-culture was attempted and the prolonged growth technique (Parker, 1936) was used here in a modified way. On the third day of incubation the coverslips were detached and washed in warm Tyrode saline (30 - 35°C) for half an hour to remove any accumulated by-products and to get rid of the excess acidity. Each coverslip ~~is~~ was then remounted on a new square slide, a drop of plasma and a trace of embryonic extract (to clot the plasma) ~~are~~ were added and the preparation sealed and incubated as before. This procedure was repeated every other day. In this way the cultures were kept alive for 10 - 12 days before they began to show any signs of degeneration.

While the hanging-drop technique was useful as a means of observing the properties of living tissues as manifested in vitro, it fell short of providing the necessary conditions for long-continued study of the same culture colonies in the same medium. The ratio of the medium to the mass of tissue in the hanging drop is so small that unless the cells are transferred to a new medium, a procedure incompatible with the aim of this experiment, the culture/

culture becomes too acid, the supply of oxygen is too limited, and soon begins to show signs of degeneration. The ideal thing would be to use culture flasks in which the colonies could be kept active for a very long time, but these were difficult to obtain (see Parker, 1938).

The success of each series of experiments depended to a great extent on the degree of asepsis and on the prevention of infection from reaching the cultures. The use of a glass hood proved to be of great help.

Between 500 and 600 preparations were made involving 30 series of cultures. 80% of the vessel-cultures were successfully grown. With each series of vessel cultures, control explants from the cardiac muscle and subcutaneous tissue from the same embryo were cultured under identical conditions.

Several series of one-to-twelve-day-old cultures were made. In every one of them at least two preparations of each day-group were fixed and stained separately to show any argyrophil and elastic fibres present in them. Control preparations were taken on the day of culturing each new series and were similarly stained. Another series of preparations containing plasma and embryonic extract only was made. It was incubated and stained in the same way as the other preparations. Great difficulty was experienced in distinguishing the details of the cultures after staining the preparations. This was due to the translucency and deep heavy staining/

staining of the thick layer of the plasma medium in which the culture was growing. All this rendered details impossible to see. This difficulty is usually overcome by embedding the whole preparation and making serial sections (Parker, 1938). This procedure was tried using different recommended techniques. Unfortunately, all failed to give a true picture of the new fibre network and its relation to the cells in a satisfactory way. Finally I had to rely on staining the preparation "in toto." The following method was very satisfactory in giving a transparent and colourless plasma medium as a background to the cells and the fibres:-

1. Place the coverslips in Tyrode saline for 1 - 2 hours.
2. Fix in 10% formol-saline for 4 - 5 hours.
3. Wash in running water for 30 minutes.
4. Immerse for 10 minutes in: -

   0.25% potassium permanganate 100 c.c.s.

   3. % sulphuric acid 6 c.c.s.
5. Wash in water.
6. Immerse for 10 minutes in 5% oxalic acid.
7. Wash in water.

This procedure appears to digest away the excess proteins in the clot without having any detrimental effect on the cells or fibres. After this proceed with the staining. Weigert's elastic stain or Orcein were used to stain the elastic tissue. It was found best to stain the cells by iron haematoxylin and not to use any counterstain./

counterstain. The Gordon and Sweets method was used to show the argyrophil fibres. Celestine blue, used after it, gave the best nuclear staining results after silver impregnation (Lendrum, 1946). The use of Mallory's triple stain as advocated by Bloom did not prove so successful.

The time allowed for each step in the staining processes, dehydration and clearing was about double that used for ordinary sections. This was done in order to take into account the thickness of the plasma clot in which the culture was embedded.

This procedure of dealing with whole preparations proved very successful. It had one great disadvantage in that they tended to wash off the coverslips at one stage of the staining process or another. No way of overcoming this could be found although several were tried. This necessarily limited the scope of the stains used and double-staining methods using both the elastic and silver stains on the same preparation were impossible to perform.

Finally, due to the use of some unplated metal instruments some of the preparations showed some black deposits after they were stained. These did in no way interfere with growth and development of the cells or fibres.

Findings:

No apparent change was noticed in or around the explants in the/

the first twelve hours after incubating the cultures. This is the period during which they become adjusted to the new environment. After this, cells began to migrate out of the explants into the surrounding medium. They soon began to multiply and spread out so that after twenty-four hours of incubation a new network of cells had surrounded the explants on all sides irrespective of their shape or size. The cells continued to multiply and spread radially out so that after forty-eight hours they formed a colony of cells, characteristic in shape (fig. 9). It was like a flat circular disc made of a single-layered network of cells. These were so arranged that their longitudinal axes were usually parallel to the radius of the colony. On the second day of incubation and thereafter, fewer mitotic figures were observed in the colony and the rate of cellular proliferation was greatly reduced. In the inner zones of the new colony, cellular division became hardly noticeable. Any multiplication of cells occurred only in the peripheral parts of the colony in order to replace any degenerating or dying cells. No further increase in the size of the colony was noticed after the third day of incubation.

The above description applied equally well to all the colonies growing from vessel, cardiac or subcutaneous tissue explants. All these tissues are mesodermal in origin and on culture give rise to similar colonies composed of cells resembling each other morphologically. Such mesenchymal cells measured 100 - 140 microns/

microns in length and because of their resemblance to the adult connective tissue cells, have been conveniently termed "fibroblasts" (Fischer, 1925, Willmer, 1935, Bloom, 1937).

In the first forty-eight hours after incubation no trace of any new elastic element was seen. Silver impregnated preparations showed the formation of delicate argyrophil fibrils. These were found extra-cellularly but in close proximity to the cells. They appeared first amongst the cells near the explant but by the end of forty-eight hours they had extended out as far as the margins of the colony and had formed into a network. This argyrophil network of fibrils was at first one layer thick but after the third day of incubation more fibrils were added to it, particularly to its inner parts. (figs. 10 and 11). There was also a simultaneous increase in the thickness of the fibres forming the network.

The first appearance of any new elastic element was seen in cultures of vessel explants after three days of incubation (fig. 12). This took the form of numerous, small, Weigert-positive granules and fibrils situated extra-cellularly. They were dispersed over a wide area around the explants, but even at this early stage the new elastic elements were regularly arranged in a certain pattern in some of the colonies (fig. 13).

The development and growth of the individual elastic fibres could best be studied in these colonies. The first elastic elements/

elements were in the form of small granules and fibrils about 1 - 3 microns in size (fig. 14). They were extra-cellular in position and like the argyrophil fibrils they developed in a slightly different plane from that of the cellular network. Very soon these granules fused together to form longer fibrils which were seen crossing over the cells in all directions (figs. 15 and 16).

As mentioned above, even as early as the first or second day after their appearance in the cultures, the new elastic elements showed a tendency to be arranged in network pattern. At this point the network was composed of many elastic granules as well as fibrils (fig. 17). As the cultures developed further so did the elastic elements of the network, especially the elastic fibrils (fig. 18). These eventually fused together to form a uniform network (fig. 19). By the fifth day of incubation the new elastic network was well formed and was composed of a single layer of elastic fibres (fig. 20). With time the fibres became thicker and better developed.

Unlike the argyrophil network, the growth of the new elastic network was confined to a certain zone surrounding the explant while the rest of the colony was free of elastic elements (fig. 21). This was invariably found in all the vessel cultures regardless of the age of the explant or the number of days it was allowed to grow. The number of the fibrils in the network increased day after day and the network became several layers thick but/

but it did not extend further out (fig. 22).

By the ninth day of incubation the new elastic network had reached its maximum growth (fig. 23). Such a network was thickest near the explant where it was made of big elastic fibres with several smaller elastic fibrils branching off from them (figs 24 and 25). As the periphery of the network was approached, the elastic fibres became less crowded and thinner (fig. 26) while the peripheral parts were made of a mixture of thin elastic fibrils and granules (fig. 27). The network was usually surrounded by a zone of elastic granules especially in the later days of incubation (figs. 28 and 29).

The newly formed elastic fibres had no connection with the elastic fibres of the explants. Bloom had suggested there was at first a migration of a few elastic fibres from the explant into the surrounding medium. This could not be confirmed in this series of experiments. Later on, after the new fibres were well developed, a few of them linked up with the cut ends of some of the explant fibres (fig. 30). But generally the cut ends of the elastic fibres of the explants remained well demarcated from the new network (figs. 13, 21, 22, 23, 33, 35 and 36).

After 10 - 12 days of incubation no more elastic element was added to the new network. Soon, the cells began to show vacuolation and signs of degeneration. The new elastic network began to break/

break up into coarse granules and the whole colony gradually disintegrated since it was not possible to keep it alive and active by using the hanging-drop technique. Odiette kept such cultures alive for one month by using flask cultures; thereafter they began to show the same signs of degeneration. Porta suggested that the breaking up of the new network was due to its digestion by proteolytic enzymes produced by the cells.

Both Bloom and Odiette reported the growth of elastic fibres in colonies of cardiac explants. This was repeated here using cardiac explants taken from different embryos 7 - 16 days old. None of the grown colonies showed any trace of new elastic tissue formation even after ten days of incubation.

In order to study the effect of cardiac contractions on the new elastic fibres in Vitro, cultures of explants containing both cardiac and aortic tissue in one piece were made. At the same time cardiac muscle and aortic tissues from the same embryo were cultured separately to act as controls. The general appearance of the new colonies of the combined explants did not differ much from those described before. The only noticeable difference was that the cardiac part of the explant continued to contract rhythmically for 8 - 10 days after incubation. During that time these contractions were exerting their effect on the cells and fibres of the colony. After ten days of incubation the contractions became irregular and finally stopped, when the colony as a whole began to degenerate.

There/

There was no appreciable difference in the size and number of the elastic fibres growing in such colonies and those growing in the colonies of the control aortic cultures (fig. 31). The only difference was that the fibres near the cardiac part of the explants were growing in the direction in which the cardiac contractions were exerting their pull.

So far only cultures made by explanting small pieces of tissues cut from the walls of the aorta or big vessels of the chick embryo have been described. The new elastic fibres appeared around the explants, surrounding them on all sides. When a complete segment of such a vessel was explanted, the new elastic fibres began to grow in a totally different pattern. After twelve hours of incubation cells began to migrate out of both cut ends of the explanted tubes but not from the sides. After forty-eight hours the whole tube was completely surrounded by cells and the colony as a whole looked very similar to those described above. The appearance of the new elastic tissue occurred also in the third day after incubation. Instead of developing all around the explant the elastic elements appeared only at both cut ends of the tube. These new elastic fibres soon began to extent out radially in fan-shape form (fig. 32). After the sixth day of incubation the new fibres began to spread in the form of an arc on the top of both ends (figs. 33 and 34). When enough time was allowed for the cultures to develop (8 - 10 days) the new elastic network was seen forming different combinations of figures especially/

especially when one or two segments of a vessel tube were explanted together (figs. 35, 36 and 37). The new network had a general tendency to develop into arcs or semicircles at each cut end. Some of these met together, thus forming complete circles of new elastic networks around the explants. Silver-stained preparations of such growths showed that the argyrophil fibres were growing in a similar manner. In all these preparations no new elastic elements were seen growing out from the sides of the explanted segments except in one case. Two segments of vessel explants were cultured together and were allowed to grow for eight days. When the preparation was fixed and stained the new elastic fibres were seen extending in arc form from both ends of one of the tubes and meeting on one side of the explants but not the other. At the same time elastic fibres were growing from the side of the explant as well and extending straight out in the direction of the new elastic circle (fig. 38). The other segment of explant did not show such a growth of elastic fibres from its ends. (compare fig. 38 with fig. 35 where two explants were also close to each other). This was the only preparation showing such an arrangement.

All the series of preparations containing only the medium showed neither argyrophil nor elastic tissue formation.

Discussion:

The following pages are devoted mainly to discussing the results/

results obtained from the tissue culture experiments. Whenever necessary, reference will also be made to some of the points raised in the preceding chapter. It must again be mentioned that this is not a discussion on the physiological and biochemical behaviour of elastic tissue in Vitro, but only a study of the formation of that tissue under experimental conditions.

In a culture of mesenchymal tissues, the cells which migrate out form a halo of growth around the central explant. This halo increases in size in the first forty-eight hours. After that there is no noticeable increase in the size of the new colony of cells. Meanwhile the rate of proliferation gradually diminishes in the direction from the outside to the centre of the colony. During this period the cells do not show any signs of resuming their functions in Vitro. This is because a dividing cell rarely functions and a functioning cell rarely divides although in a developing organism, one cell may be functioning and its next-door neighbour still dividing (see Willmer, 1935). After this period of unorganised growth, the cells may begin to show signs of proceeding to perform the same functions as they did in the living organism. This inherent capacity to function shows itself only if the culture is done under favourable conditions. These were provided by using the prolonged culture technique.

Once the period of organised growth begins, the new elastic elements begin to form. The first elastic granules were seen to be/

be extra-cellular in position. No direct connection could be found between them and the cells around them. There was no trace of any Weigert-positive substances inside the cytoplasm of the cells, as was suggested by Odiette. The new elastic elements formed in a slightly different plane from that in which the cells were lying.

We have already mentioned the theory denying the participation of the cells in the process of fibre formation (Baitsell, Wolfe). The cultures described here were made under ordinary conditions necessary for the maintenance of any type of embryonic cells in Vitro. The new growth was under no special physiological influences such as are present in the living embryo. Yet such cultures showed a formation of a new elastic network. The fibres of this network were not seen developing in the incubated control media or in parts of the cultures where the cells were not present. In fact, the new elastic network only formed in a certain zone of the colony (vide infra). This excludes the possibility that the medium, per se, and without cellular action, is responsible for the fibre formation.

The explanted pieces of vessel tissue were formed mainly of cells and already-formed elastic fibres. It might be suggested that the new elastic tissue is formed from a re-arrangement of the elastic fibres present in the explant. These fibres did not show any change in their number, size or position. Even after ten days of culture, their appearance was very similar to that of the/

the control explants taken from the same embryo. Furthermore, in some of the older cultures, the total amount of the new elastic tissue appeared to be more than that which could be formed from the elastic tissue of the explanted piece even if the latter had completely changed into the former. Porta (1930) succeeded in growing elastic fibres from explants of three-day-old embryonic vascular tissues when at that stage no trace of any elastic element had yet developed in the embryo.

All these findings, coupled with those found in the preceding chapter, point to the conclusion that the elastic fibres are formed extra-cellularly, but that the presence of cells is necessary for their formation and that in their absence no elastic fibres could be formed, in either the embryo or in tissue culture.

We next pass on to the question of whether this cellular activity of elastic tissue formation was inherent in special cells only, or not. Those who deny this, build their argument on the absence of any morphologically distinct cells performing that function. We have already explained how certain cells can be chemically differentiated to perform a special function, while they are not histologically differentiated.

The various mesenchymal cells taken from the same embryo and cultured under identical conditions yielded the same type of colony composed of the so-called "fibroblasts." The appearance of the individual cells and some of their characteristics have already/

already been mentioned. It has been shown by Parker (1933) that the fibroblasts, grown when culturing explants of mesenchymal origin, as a group, comprise many cell-races. They have certain general properties applicable to all of them but at the same time they retain certain inherent capacities for development along definite lines, i.e., they are only partially de-differentiated. Once they are given the necessary medium and time, each cell race manifests certain functional properties according to the particular organ or tissue from which the explant was derived. It follows, then, that the special products produced by a given cell colony in Vitro reflect the characters of that part of the organism from which the explant was taken and the products are the same as those which the cells were forming in Vivo at the time of the explantation. That is why the fibroblasts originating from aortic and vessel explants of 8 - 12 day-old chick embryos were the only ones capable of producing elastic tissue in Vitro. None of the other explants showed this.

It might again be argued that the general connective tissue cell could form elastic tissue under the influence of certain physiological needs of the organism. As mentioned before, no such influences or needs exist in tissue culture. The cultured cells only manifest the properties inherent in them, as conditioned by external influences. Such external influences were present in all the cultures since a standard technique was used. So it follows that the specific function of elastic fibre formation is primarily/

primarily present as an inherent property in certain mesenchymal cells in the walls of the embryonic vascular system. These cells have become chemically but not morphologically differentiated. Only these cells are capable of forming elastic tissue in Vitro. Whether such cells should be called "elastoblasts" or "Elastic producing fibroblasts," both in the living organism and in culture, is a matter of personal preference.

The elastic network was limited to a certain zone of the colony. This zone formed the inner third of the area of the whole colony. The cells in this zone did not differ in appearance from the other cells of the colony. It appears that a colony of cells in a hanging-drop medium differentiates into three functional zones:-

1. An inner vegetative zone in which the cells differentiate to perform their function.
2. An intermediate zone of proliferating cells, and
3. An outer zone of dissociated cells forming the margins of the colony (Fischer, 1946).

It appears that the cells which migrated out of the explant and formed the first zone retained the ability of elastic fibre formation better than their progeny which migrated further out. That is why the new network was thicker nearer the explant and became thinner as it extended out. The rate of cellular proliferation (which is incompatible with function) diminishes from the/

the outside to the centre of the colony. This applies only to hanging-drop cultures. In flask cultures, the zone of functioning cells was much wider and so was the formed elastic network (see Odiette).

The new elastic granules fused together to form small fibrils, which in turn fused together to form longer ones. This process continued until the whole elastic network was formed. The increase in the thickness of the individual fibril appeared to be through the deposition of more elastic element directly on them or in the form of small elastic granules which gradually merged into the substance of the fibres.

A minor point that was cleared up by the tissue culture experiments was the authenticity of the elastic granules. Many authors believed them to be parts of elastic fibres cut transversely. No sectioning was performed on any of the culture preparations, yet the elastic granules were very much in evidence.

The relationship between the argyrophil and collagen fibres has been mentioned before. Both Levi and Bofill-Deulofeu have shown that in all the colonies of mesenchymal cells (fibroblasts) there are laid down argyrophil fibres between the cells (Willmer). This argyrophil-fibre formation is one of the general characteristics of all types of mesenchymal cells and can still be shown even after many subcultures (Fischer). We have already seen those fibres developing in all the colonies of vessel, cardiac or subcutaneous/

subcutaneous tissues. By the time the first elastic granules were beginning to appear, the argyrophil fibrillar network was well developed, extended to the margins of the colony and was not restricted to a limited zone like the elastic network. Bloom found no relation between the argyrophil and elastic networks. It is unfortunate that no double staining similar to that done on the embryo sections, could be made on the culture preparations. Therefore the exact role that these argyrophil fibres play in elastic tissue formation cannot be fully ascertained.

It might be suggested that the argyrophil fibres (which are formed by the same cells as these that make the elastic substance) form a framework on which the elastic element is deposited. This would also explain why the first elastic elements were seen arranged in pearl-string fashion. Doljanski and Roulet (1933) and Stearns (1940) favour the conception that certain substances are secreted from cells and that these substances act on the albumoids in the surrounding medium and change them into collagen. The same probably happens in the case of the elastic fibres. The special cells or elastoblasts secrete certain substances which by acting on the surrounding albumoids change them into elastic element. This element by virtue of surface tension is deposited on the existing argyrophil fibrils in the form of small globules. It is for the same reason that the first coat of paint, applied on thin wires, takes the form of globules. (For full discussion and references of this phenomenon see J. Z. Young, 1945). Later

on/

on as more elastic element is deposited and as the element gradually merges into the substance of the argyrophil fibrils a thicker and more uniform elastic fibre is formed. This is only a tentative suggestion and will need further experimental evidence.

It has already been mentioned that the first appearance of the argyrophil and elastic fibres in the walls of the aorta in the chick embryo coincided in time. Since the same fibres took both stains it was suggested that the early elastic fibres had argyrophil properties (see P 28). It was also shown that the cells from the aortic wall had the ability to form both argyrophil and elastic fibres in tissue culture. These cells formed the argyrophil fibres as they began to spread out and proliferate, but it took them three days to differentiate and start forming the elastic element. This is a general rule in all tissue culture explants (a stage of proliferation or unorganised growth followed by a stage of differentiation or organised growth). This difference in time is not so noticeable in the chick embryo and the cells proliferate and differentiate very rapidly. That is why we find the argyrophil fibrils and the elastic elements appearing at the same time. Later on as the elastic elements became more concentrated in the fibres, they gradually lost their ability to take the silver stain.

Bloom was of the opinion that the cardiac contractions were very important in influencing the development of the elastic fibres/

fibres in tissue culture. This was not confirmed in the series of cultures. The only effect the contractions had was that they changed the direction of the fibres' growth.

The development of the arc-shaped elastic networks when complete segments of vessels were explanted, is difficult to explain. No such type of growth was found mentioned in the literature. All the migration of cells out of such explants occurred only at the cut ends since they could not grow out of the uncut sides of the segments. They spread out in a fan-shaped manner at both ends and it is probable that the first generation of cells continue growing in that peculiar configuration and thus the elastic fibres are found later in the same position.

Conclusions:

The following general conclusions can be drawn regarding the formation of elastic tissue in embryonic tissues both in Vivo and in Vitro:

1. The ~~first~~ elastic element was first seen extra-cellularly in the form of granules.
2. Special cells chemically but not morphologically differentiated are responsible for that formation.
3. The argyrophil fibres act as a framework on which the elastic element is deposited.

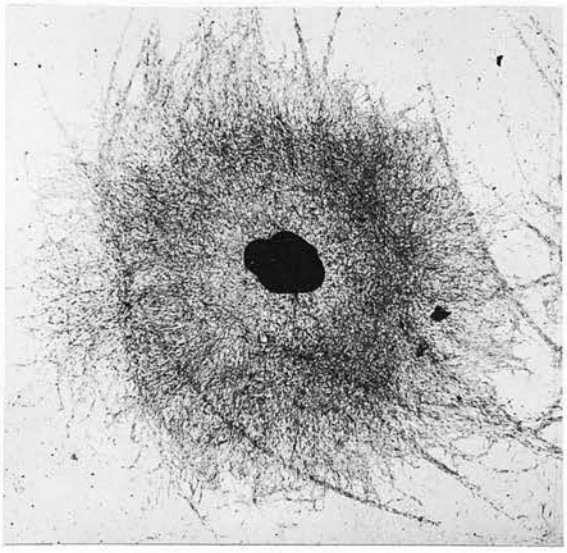

Fig. 9.

48-hour old colony of an aortic explant.
Weigert - Iron haematoxylin. x 8.

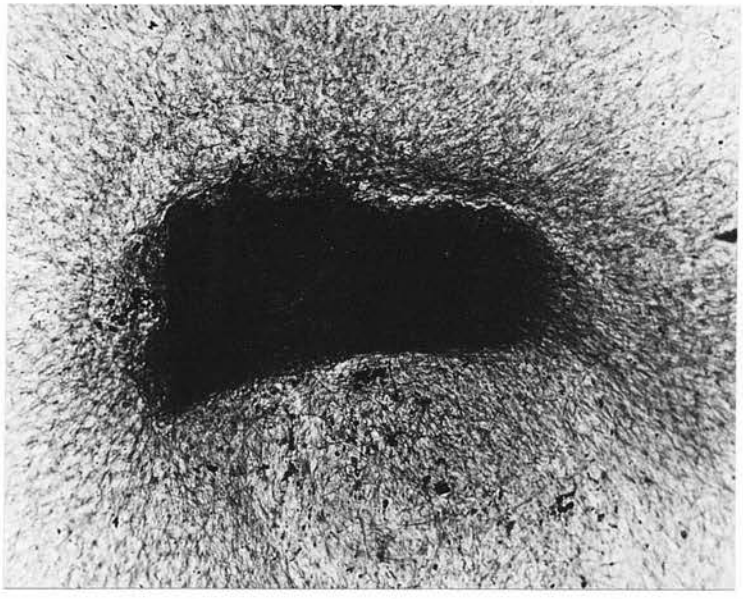

Fig. 10.

5-day old culture of an aortic explant showing the argyrophil network in the colony. The network is thicker nearer the explant.
Gordon & Sweete - Celestine blue. x 50.

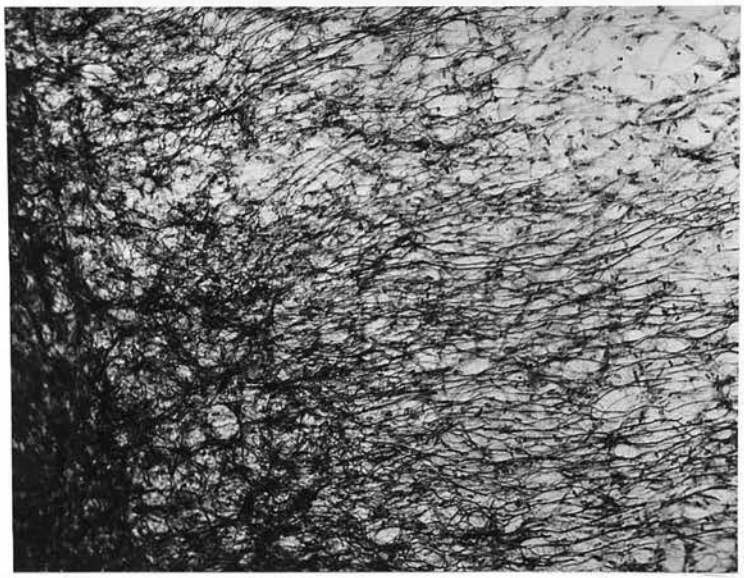

Fig. 11.

High-power view of the argyrophil network seen in Fig. 10.
Gordon & Sweete - Celestine blue. x 120.

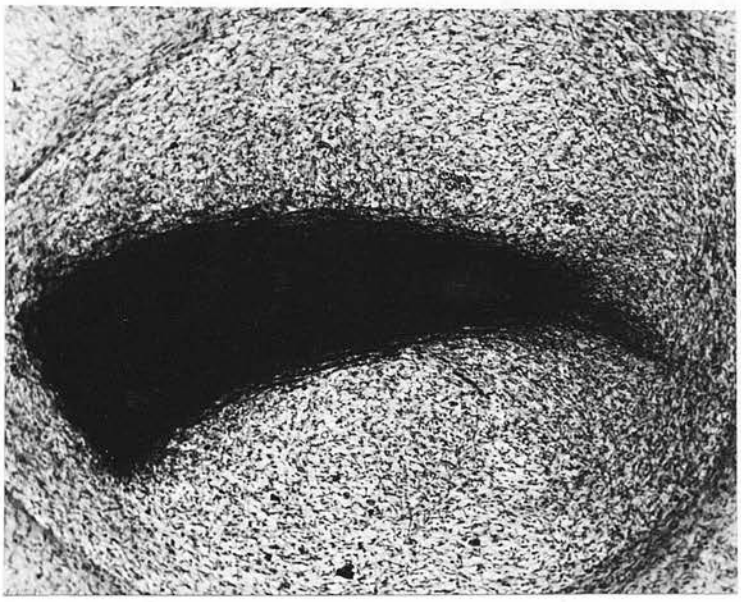

Fig. 12.

3-day old culture of an aortic explant. Elastic granules are beginning to appear around it.
Weigert - Iron haematoxylin. x 50.

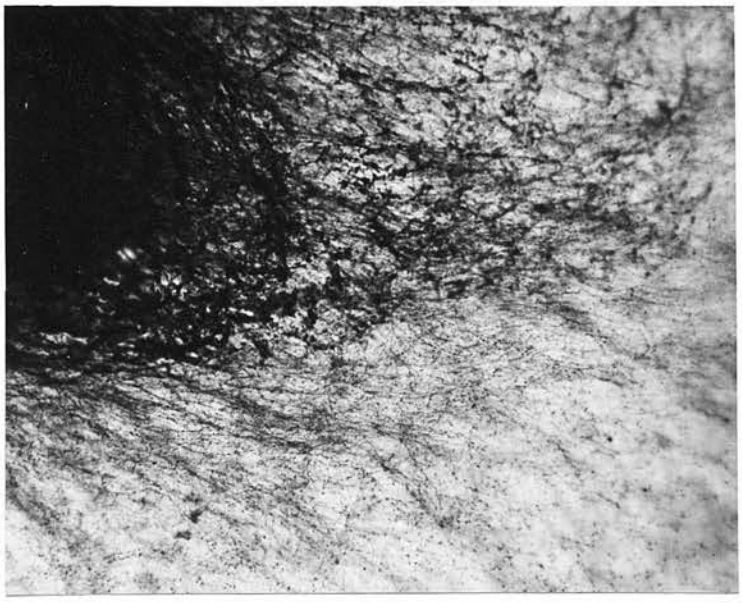

Fig. 13.

High-power view of one end of the explant seen in Fig. 12. The elastic elements are arranged in a network.
Weigert - Iron haematoxylin. x 240.

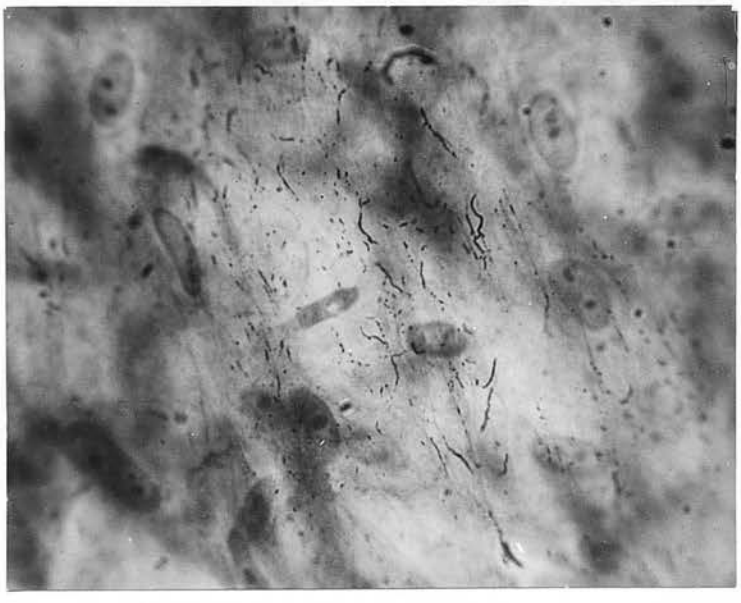

Fig. 14.

Part of a 3-day old colony of an aortic explant. The elastic granules and fibrils are extra-cellular in position. The cellular outlines are not well seen since they lie in a different plane.
Weigert - Iron haematoxylin. x 800.

Fig. 15.

3-day old aortic culture. The elastic fibrils are more prevalent in this part of the colony.
Weigert - Iron haematoxylin. x 800.

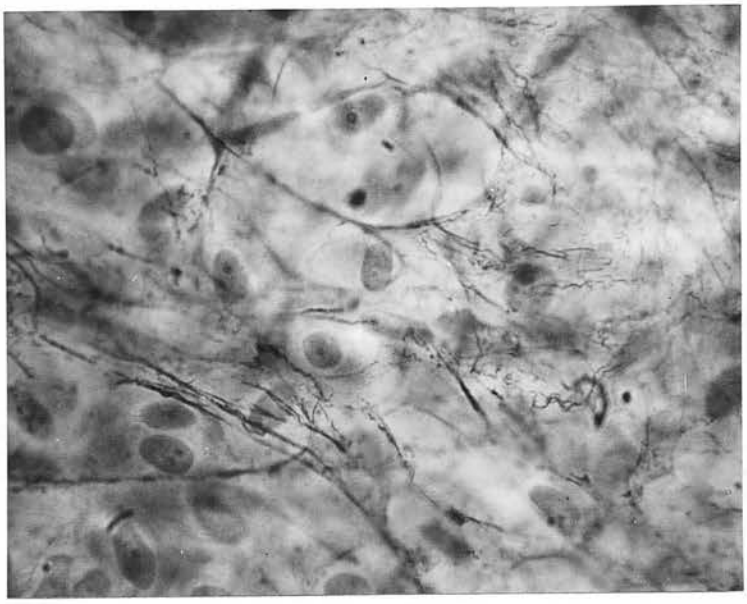

Fig. 16.

Another part of the same colony as shown in Fig. 15.
Weigert - Iron haematoxylin. x 800.

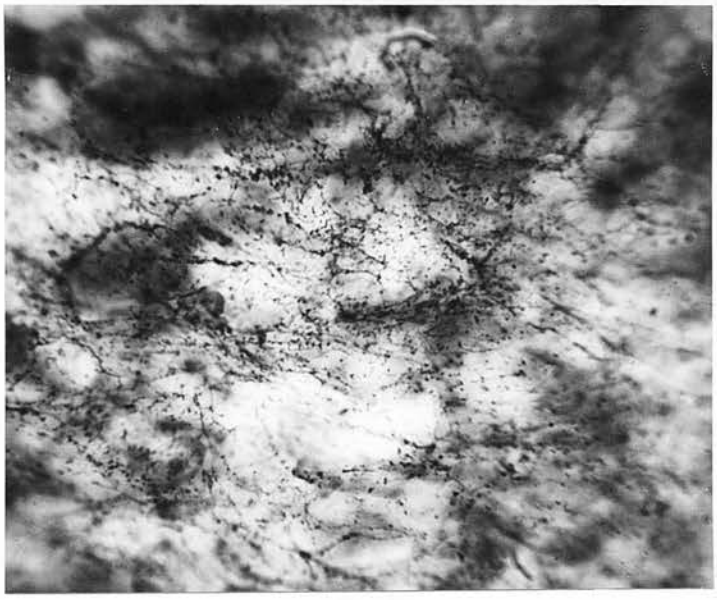

Fig. 17.

High-power view of an area near the explant shown in Fig. 13. The new elastic granules and fibrils are arranged into a network.
Weigert - Iron haematoxylin. x 800.

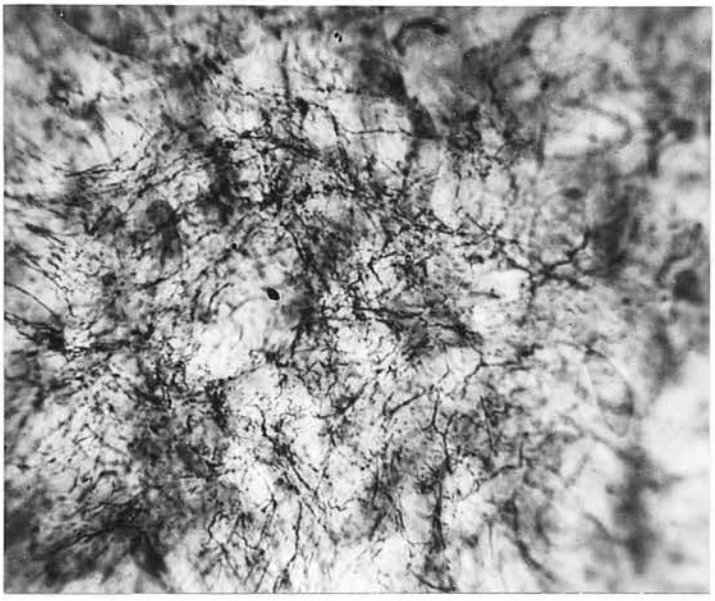

Fig. 18.

4-day old culture of a vessel explant. The new elastic network is now predominantly fibrillar rather than granular.
Weigert - Iron haematoxylin. x 800.

Fig. 19.

5-day old culture of a vessel explant. The new elastic network is now uniformly fibrillar.
Weigert - Iron haematoxylin. x 800.

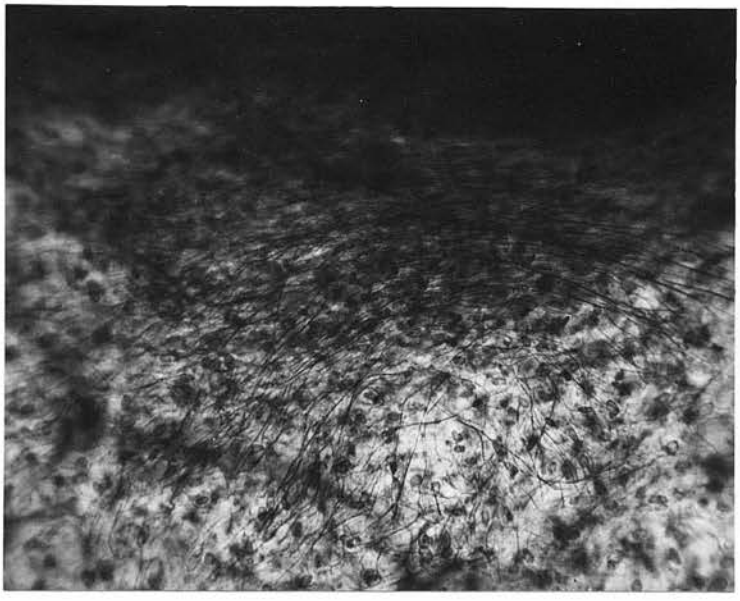

Fig. 20.

5-day old culture of a vessel explant showing the full extent and appearance of a part of the new elastic network.
Weigert - Iron haematoxylin. x 240.

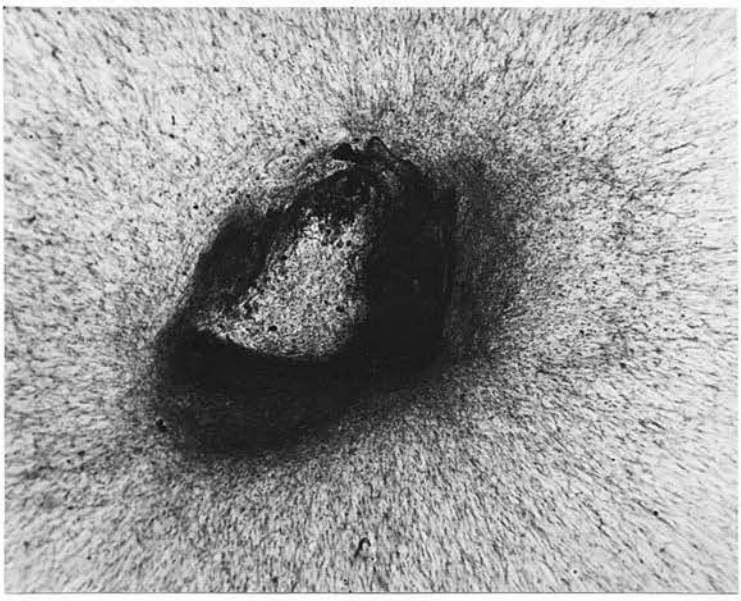

Fig. 21.

7-day old aortic culture. The new elastic network is formed in a limited zone of the colony. Fewer elastic fibres have grown in the area next to the damaged part of the explant.

Weigert - Iron haematoxylin. x 50.

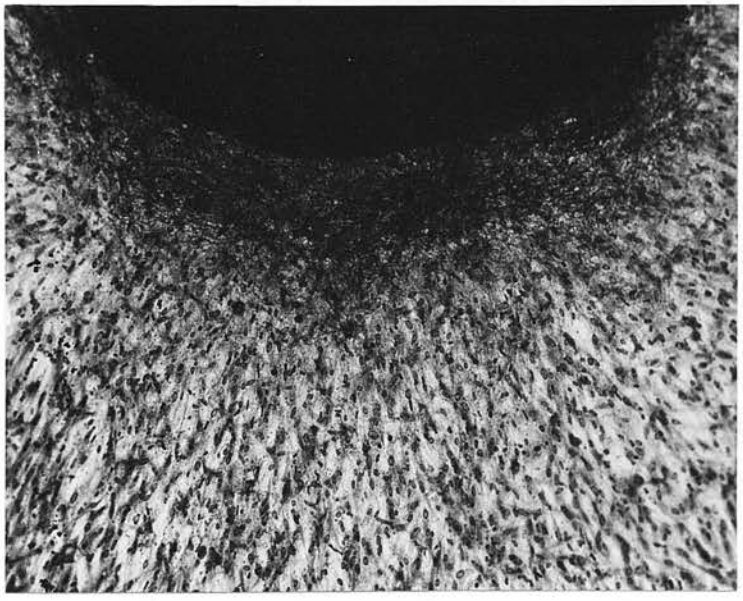

Fig. 22.

High-power view of the culture shown in Fig. 21. The new elastic network becomes thinner as it extends further out.

Weigert - Iron haematoxylin. x 100.

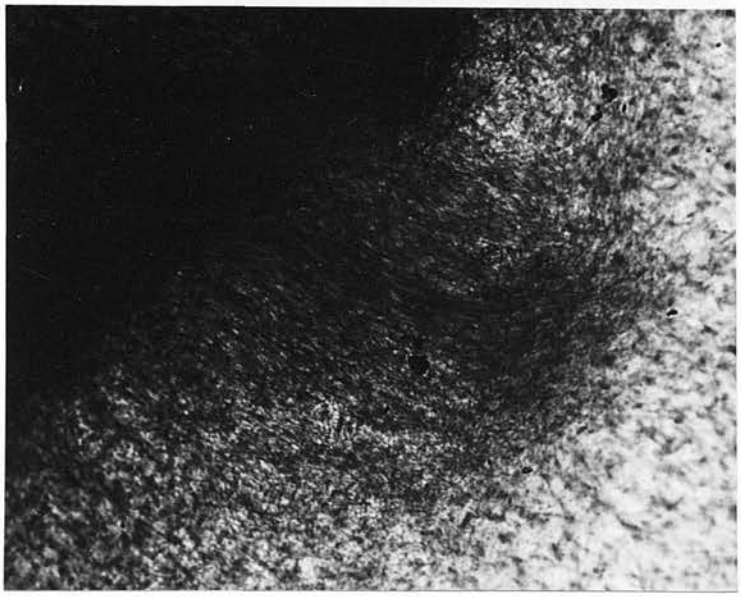

Fig. 23.

The new elastic network in a 9-day old culture of a vessel explant.

Weigert - Iron haematoxylin. x 100.

Fig. 24.

High-power view of the culture shown in Fig. 22. It was taken near the junction of the new elastic network and the original explant.

Weigert - Iron haematoxylin. x 240.

Fig. 25.

A higher magnification showing the details of the elastic fibres seen in Fig. 24.
Weigert - Iron haematoxylin. x 480.

Fig. 26.

A field in the middle region of the new elastic network shown in Fig. 22.
Weigert - Iron haematoxylin. x 800.

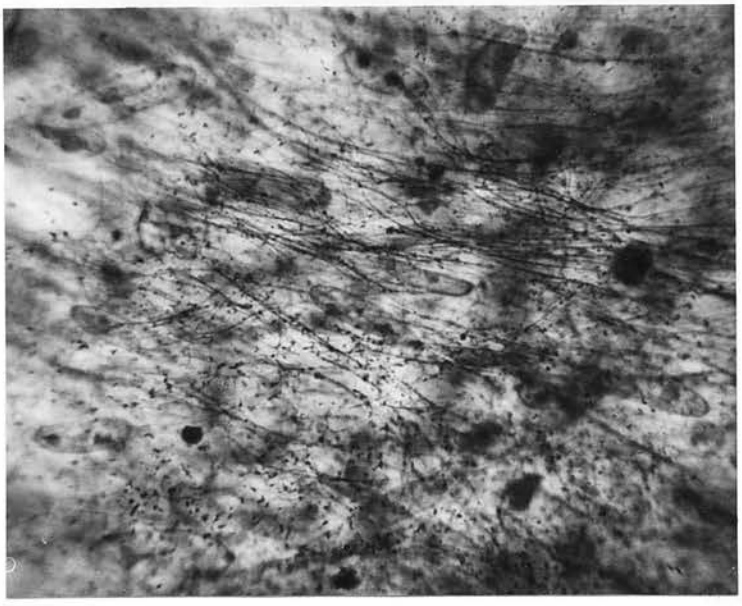

Fig. 27.

A field near the periphery of the elastic network shown in Fig. 22. The elastic fibres are very thin and the elastic granules are numerous.
Weigert - Iron haematoxylin. x 800.

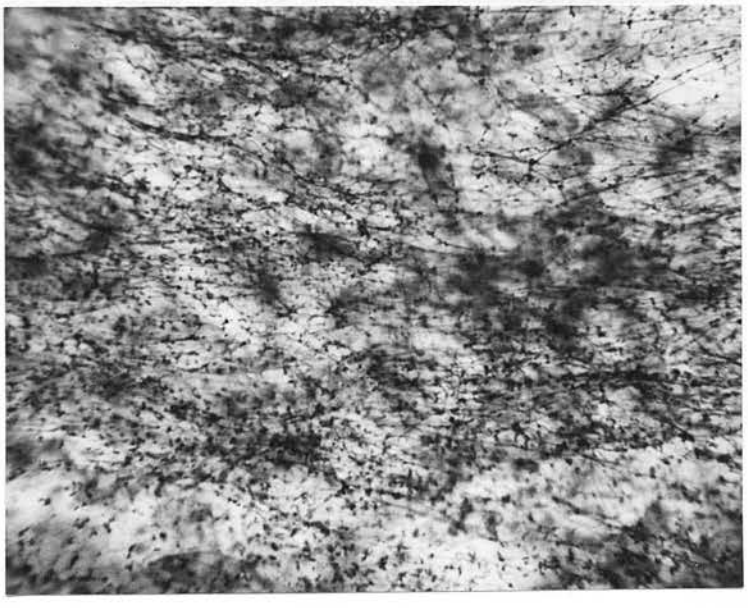

Fig. 28.

A field taken at the margin of the elastic network shown in Fig. 22. The elastic granules are very numerous.
Weigert - Iron haematoxylin. x 800.

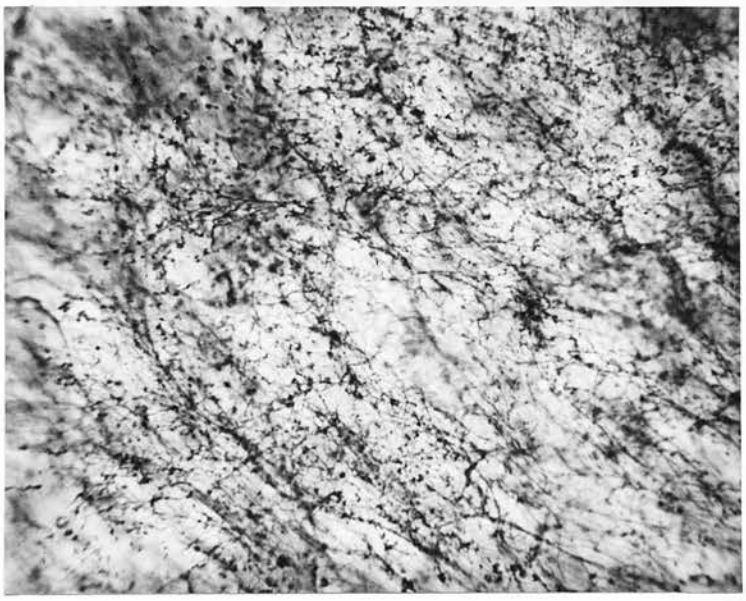

Fig. 29.

Another field in the same zone shown in Fig. 28.
Weigert - Iron haematoxylin. x 800.

Fig. 30.

5-day old culture of an aortic explant showing the link between the new and old elastic fibres. Note the different direction which each network follows.
Weigert - Iron haematoxylin. x 480.

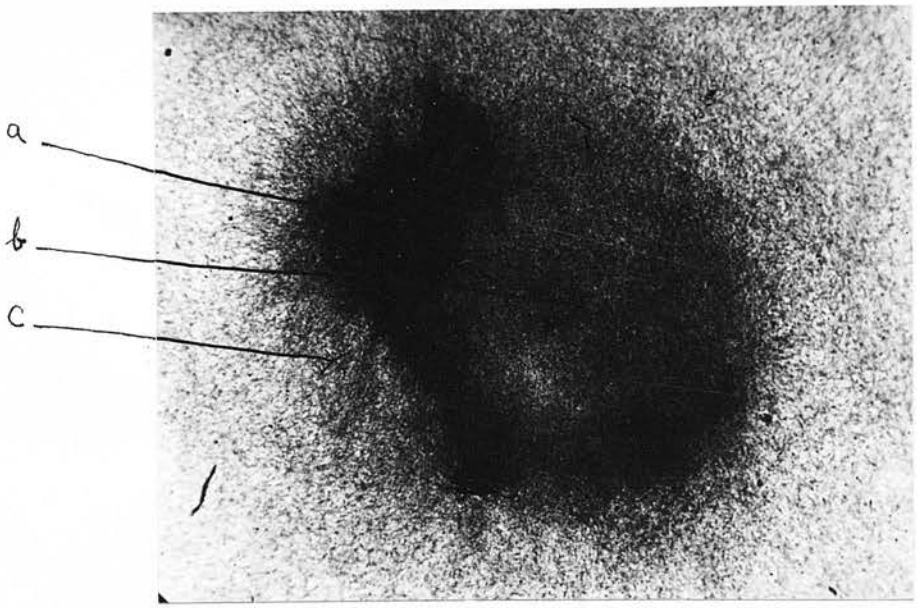

Fig. 31.

6-day old culture of an aortic-cardiac explant. No elastic fibres are seen around the cardiac part of the explant.
a. aortic part. b. cardia part. c. new elastic network.
Weigert - Iron haematoxylin. x 50.

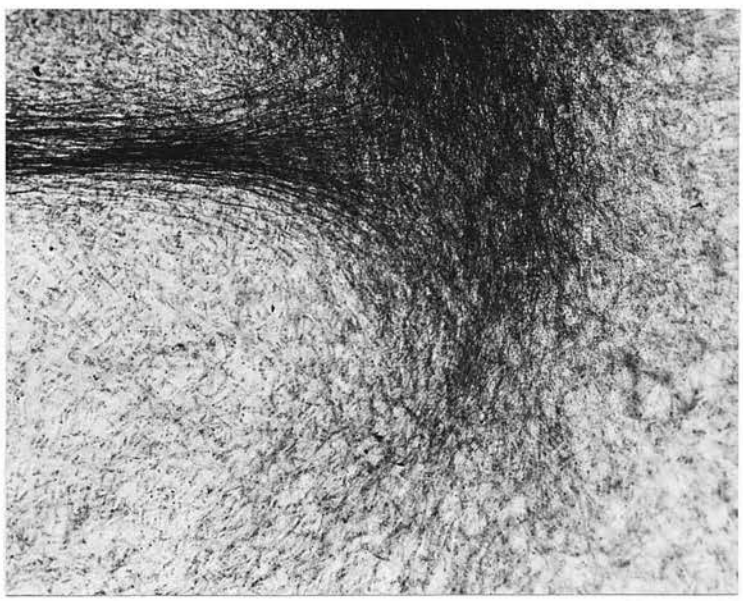

Fig. 32.

5-day old culture of a vessel segment. The new elastic network is developing in a fan-shape form at one end of the explant. No growth of elastic fibres is occurring at the side.
Weigert - Iron haematoxylin. x 100.

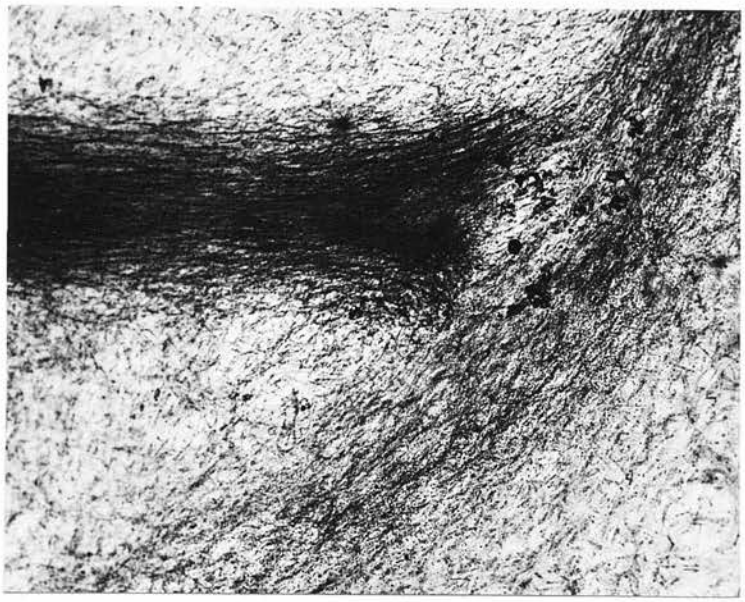

Fig. 33.

Part of Fig. 36 showing the arc shape the new elastic fibres have taken.
Weigert - Iron haematoxylin. x 100.

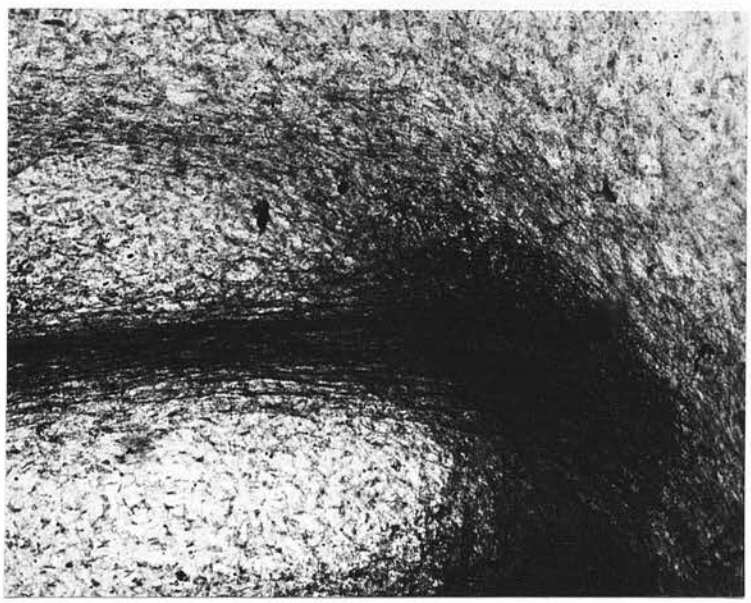

Fig. 34.

Part of Fig. 35 showing the arc shape the new elastic fibres have taken.
Weigert - Iron haematoxylin. x 100.

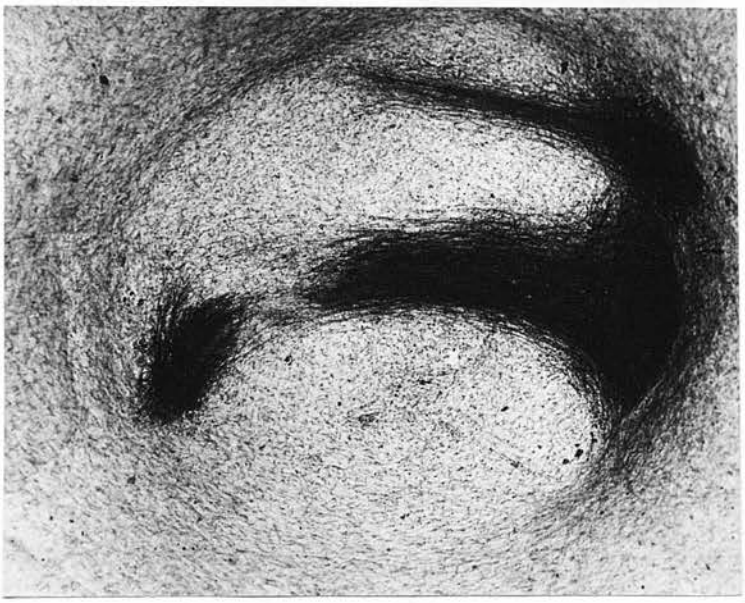

Fig. 35.

6-day old culture of three vessel segments. The new elastic network tends to surround the explants in the form of a circle.

Weigert - Iron haematoxylin. x 50.

Fig. 36.

8-day old culture of 3 vessel segments. Note again the tendency of the new elastic network to form a complete circle around the explants.

Weigert - Iron haematoxylin. x 25.

Fig. 37.

9-day old culture of several vessel segments. Note the curious arrangement taken by the developing elastic networks. In the lower left-hand corner a piece of the aorta shows the growth of the elastic network all around it.
Weigert's stain. x 25.

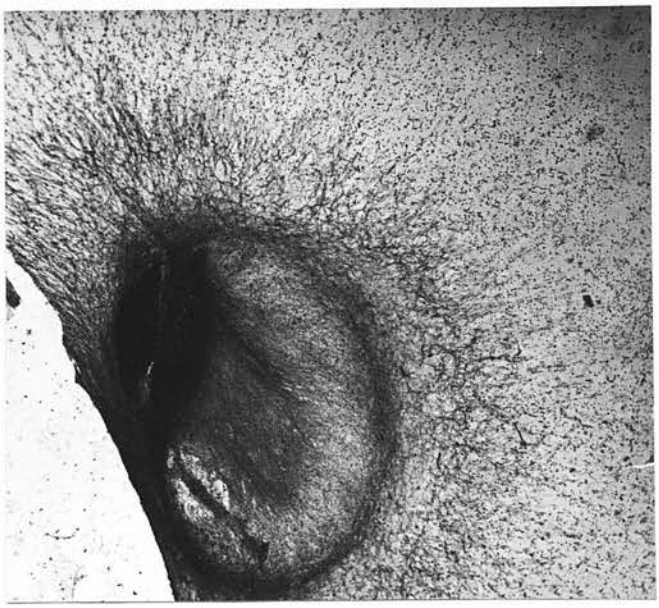

Fig. 38.

8-day culture of two vessel segments. The elastic networks growing at each end of one segment have met on the undamaged side of the colony. Elastic fibres are growing out from the side of the segment as well.
Weigert - Iron haematoxylin. x 25.

CHAPTER IV.

THE DEVELOPMENT OF ELASTIC TISSUE IN CANALISING BLOOD CHANNELS.

THE DEVELOPMENT OF ELASTIC TISSUE IN CANALISING BLOOD CHANNELS

The elastic tissue, like other connective tissues in the body, shares in the reaction to injuries and diseases of the adult organism. Under certain circumstances many new elastic fibres are formed. One of the best examples is seen in the new elastic coats developing around canalising channels of arteries affected by Thrombo-angiitis obliterans and arteriosclerosis of the lower limbs.

The aetiology and initial pathological changes occurring in both these conditions are not fully understood. While it is generally accepted that thrombosis in vessel segments is the cause of occlusion in Thrombo-angiitis obliterans (Buerger, 1924 and 1939, Allen et al 1946), others believe that it is caused by the proliferation of the intima (Krompecher, 1928 and 1930, Dürck, 1930, Gruber, 1930, Jäger, 1932, Popken, 1936). In arteriosclerosis of the lower limbs the main lesions are degeneration of the media and intimal atheromatous lesions. Whether the atheroma starts with degeneration or proliferation is not settledyet (Cowdry, 1933, Leary, 1941). Thrombosis is an almost invariable accompaniment of these changes, hence the name "Thrombo-Arteriosclerosis obliterans" suggested by Hines and Baker (1940).

Because of these debated points, it was sometimes difficult to interpret the sequence of events that led to the obliteration of/

of the vessel lumen in these cases. By the time the clinical manifestations appeared, many of the initial pathological changes had disappeared, leaving a picture which was the resultant of several successive lesions. Sometimes more than one kind of lesion contributed to the final state. The course of both diseases is greatly modified by the development of an efficient collateral circulation. When this finally fails to cope with the blood supply to the tissues of the limb, gangrene supervenes.

The organisation of thrombi occluding vessel lumina has been fully studied. It is generally agreed that the vascular supply to the organising thrombi come by capillaries originating in the vasa vasorum and penetrating the vessel wall to invade the thrombi (Heucking and Thoma, 1887, Buerger, 1924, Tannenberg, 1927, Borst, 1936, Gery et al, 1939). The thrombi are softened and replaced by vascular connective tissue. As this becomes more fibrous, it contracts, thus allowing for the formation of small and large blood spaces which give the arterial lumen its characteristic cribriform appearance. In still older lesions some of the spaces become surrounded by elastic coats. Accordingly the following types of blood channels may be found in an old occluded vessel segment:-

1. Small capillaries scattered in the lumen.
2. Blood spaces or sinuses lined by endothelial cells only.
3. Elastic coated channels running in the longitudinal axis of the lumen.

Very/

Very little was found to have been written about these elastic-coated channels, how they formed and why they acquired elastic coats. Virchow (see Tannenberg) showed that the channels present inside an organised thrombus did not communicate directly with the patent functioning parts of the vessel lumen above and below the thrombosed segment. We know that vessels in which the blood is flowing under some pressure develop elastic coverings. On the other hand, it is agreed that the vascular spaces derive their supply from the ingrowing capillaries from the vasa vasorum (vide supra). This does not explain the development of the elastic coats. It was hoped that by studying the circulation inside occluded vessel segments an explanation might be found.

Materials and Methods:

A histological study of the vessels in twenty-six gangrenous lower limbs was made. Eighteen cases were due to Thrombo-angiitis obliterans and eight cases due to Arteriosclerosis. Representative vessel segments were taken from the popliteal artery, upper and lower parts of tibial arteries, the dorsalis pedis and plantar arteries. Thirty segments that showed complete occlusion and recanalisation were serially sectioned at five microns thickness. Every fifth section was mounted and stained by Weigert-Haematoxylin - Van Gieson. A few representative sections were stained by Haematoxylin - Eosin. Between one hundred and fifty and two hundred sections of each vessel segment were examined.

Eight/

Eight cases are described in detail in the appendix to this chapter. They were selected to show the various aspects of the occlusive processes described in the chapter. All the microphotographs were taken of sections stained by the Weigert - Haematoxylin-Van Gieson stains. It was surprising how much additional information was obtained from elastic-stained sections, as compared with those stained by the usual stains. They show all the details of the changes in the vessel walls and the stages of obliteration and recanalisation of the lumen.

Findings:

The lumina of the large vessels of the limb were patent or occluded only by recent thrombosis. The medium sized vessels were usually the ones occluded in both Thrombo-angiitis obliterans and Arteriosclerosis, particularly the lower parts of both the anterior and posterior tibial arteries. The smaller vessels were usually patent or occluded by more recent thrombosis than that in the medium sized vessels.

Thrombo-angiitis obliterans: The changes in the vessel walls of the occluded segments were those generally described in this disease. The internal elastic lamina was well preserved and in some cases proliferated. It was deficient only in places where it was traversed by some vascular channels running between the lumen and the media. The media showed slight fibrosis, vascularisation and round cell infiltration. The adventitia was usually extensively fibrosed and infiltrated with foci of round cells.

The lumen in the occluded segments was obliterated by old fibrosed connective tissue. A varying amount of blood pigment indicated that the connective tissue had replaced a thrombus. The vascular spaces consisted of the three types described above.

When the small capillaries in the lumen were followed in serial sections they were found to collect together and form one of two types of vascular channels.

1. They collected into blood sinuses which were lined by endothelial cells only. (figs. 39 - 42). These sinuses varied in diameter between 200 - 500 microns, but were not more than 200 - 700 microns in depth. They usually collected in the regions where the branches from the main vessels were given off, and divided again into smaller sinuses. These sinuses ran transversely in the lumen of the vessel towards the periphery. They penetrated the internal elastic lamina, traversed the media and ended in the vasa vasorum of the adventitia. Such vasa could be followed in serial sections till they joined a vein (figs. 45 and 46).

2. The rest of the capillaries collected together and eventually ended in small elastic-coated blood channels. These gradually joined together forming larger ones. They ran in the longitudinal axis of the vessel lumen and invariably ended in one of the side branches (figs. 48 - 54). Other elastic-coated channels were not seen to divide but always ended by joining a side branch (see pictures of Cases I - IV in Appendix).

Serial sections of occluded vessel segments that had no side branches/

branches did not have any elastic-coated canalising channels in their lumina although they were full of vascularised organising thrombi.

The elastic-coated channels were either patent (figs. 39-44 and 55-59), or were filled with an organised thrombus-tissue of a more recent nature than that filling the main vessel lumen (figs. 48-52 and 61-66). Recanalisation was seen beginning in some of these occluded blood channels (figs. 50 and 62).

The elastic coats surrounding such channels were made either of thin elastic fibres (figs. 39-44) or of several layers of thick elastic fibres (figs. 48-53, 55-59 and 61-66). The innermost elastic coat lining the channel was continuous with the elastic lamina that lined the lumen of the side branch it joined (figs. 41, 52, 57 and 61). At the site of the junction of the channel and the side branch there was a cushion-like thickening of the intima made of several layers of elastic fibres (figs. 41, 52 and 57).

The side branches with which the channels were connected had relatively well preserved walls. Near the main vessel they showed one of the following pictures:-

1. They were fully patent with intact walls (fig. 57).
2. They were partially occluded by the same kind of tissue which filled the main vessel lumen. The remaining part of the lumen was patent and lined by an elastic coat which was continuous with that lining the channels in the main lumen (figs. 39-42 and 64-65).

3./

3. The walls showed the same changes as those presented by the main vessel. The remaining lumen was filled with the same kind of recently organised thrombus that was filling the elastic-coated channels with which they were continuous (figs. 52 and 61).

All these side branches were connected peripherally with smaller arteries that were patent and filled with blood.

In some of the segments, a successive series of thromboses had occurred in the lumen (figs. 55-59). A mural thrombosis had taken place first, which was organised and lined by a new elastic lamina. Later on another thrombosis occurred and was recanalised by two channels originating from the two side branches.

Still another picture was presented by segments that usually showed older types of lesions. The peripheral half of the lumen was replaced by a concentric fibrous layer containing a skein of elastic fibres and fibrils. This layer was limited externally by the internal elastic lamina of the main vessel and internally by the new elastic coat surrounding the canalising channel (figs. 48-53 and 61-66). Such a thickening was very patchy in distribution and extended for about $\frac{1}{2}$ - 1 inch along the lumen of the vessel. It gradually became thinner until it disappeared and the lumen was again filled by organised thrombus-tissue only. Sometimes this layer was present in the side branches for some distance along their length (figs. 53 and 61).

This layer was found only in some of the vessel segments affected/

affected by Thrombo-angiitis obliterans but not with Arteriosclerosis. Krompecher and others (vide supra) thought that this was due to intimal proliferation of the fibro-elastic layer of the intima which is part of the initial change in Thrombo-angiitis obliterans. Buerger (1924) and Leriche (1946) thought that it was due to localised arteriosclerotic intimal proliferation superimposed on an existing thrombo-angiitic lesion. As mentioned before, the lesions seen in the vessels of amputated limbs are the resultant of several changes acting over a long period of time and it is difficult to be certain of the sequence of events.

Arteriosclerosis:

Several parts of the medium sized vessels were occluded by organised thrombi, a few by the formation of plaques or both. (Thrombo-arteriosclerosis obliterans). It is uncertain what role intramural haemorrhage (Winternitz et al 1938) or mural thrombosis (Duguid, 1946) plays in formation of the arteriosclerotic plaques.

Segments occluded by either type of tissue showed recanalisation by elastic-coated channels.

1. In segments occluded by organised thrombus tissue, the sequence of events followed lines similar to those described under thrombo-angiitis obliterans. The small capillaries collected either into non-elastic blood sinuses or into elastic-coated channels. The blood sinuses were shallow and lined only by endothelial cells. They/

They eventually penetrated into the media and joined some of the vasa vasorum (figs. 69, 70 and 76). The elastic-coated channels ran in the longitudinal axis of the lumen, collected together and ended by joining one of the side branches coming off the main vessel (figs. 68-70 and 72-78). The elastica surrounding these channels was continuous with that surrounding the lumina of the side branches which they joined (figs. 69, 70, 74 and 77). The channels were either fully patent (fig. 77) or occluded by some intimal proliferation and thrombosis of a more recent nature than that occupying the main vessel lumen (figs. 70, 74 and 75).

The side branches were also either fully patent (fig. 77), partially occluded (figs. 69, 70 and 75), or occluded by the same kind of recently organised thrombus that occupied the elastic-coated channels (fig. 74). All these side branches were peripherally connected with fully patent smaller vessels.

2. The atheromatous plaques that completely filled some of the vessel segments were made of several layers of fibrous and hyaline tissue separated from each other by degenerating elastic fibres and granules (figs. 82 and 88). Small elastic-coated channels were seen running along the longitudinal axis of the lumen and they joined one of the side branches of the main vessel (figs. 81, 84 and 89). Such side branches were either patent and had normal walls (figs. 81 and 85) or were affected by the same arteriosclerotic process (fig. 89). These side branches were/

were connected peripherally with smaller, fully patent vessels.

The part of the vessel wall nearer the canalising channels was relatively well preserved, compared to the rest of the wall. This suggested that these side branches helped in the nutrition of the vessel wall.

In Arteriosclerosis also, the occluded vessel segments that had no side branches showed no recanalisation by elastic-coated channels.

Discussion:

The examination of occluded vessel segments affected by thrombo-angiitis obliterans and arteriosclerosis showed that all the elastic-coated channels found in their lumina followed the same steps of development. It appears that when the main arterial lumen is occluded in either of these diseases, the small side branches are not involved or only partially so. The arterial supply to the limb is carried by the collateral circulation. By using different methods, several authors have proved that the arterial supply to the limb through the collateral circulation may be adequate even when some parts of the main vessels are completely occluded (Lewis and Reicher, 1926, Horton, 1930, Allen and Camp, 1935). Buerger (1924) stressed the fact that when the main vessels are occluded, some of the patent smaller branches, coming off the main occluded vessels, participate in the collateral circulation. In these vessels the blood flow may be running in the reverse/

reverse direction to that in which it used to run before the occlusion of the main vessel. This was further proved by some experiments done in this department (vide infra). The patent side branches coming off an occluded vessel segment were found to form part of the collateral circulation and were full of blood. These side branches carried arterial blood under pressure, but the flow came to a standstill near the origin of the side branch as its way was blocked by the thrombus. When the thrombus began to soften and organise, the arterial blood stream forced a passage through it. The formation of this passage is helped or retarded by many factors, e.g., the efficiency of the collateral circulation, the pressure of the blood inside it, the degree of patency of the side branch, etc. Thus in the segment shown in figs. 39 - 44 the blood channel ended blindly, probably due to the partial occlusion of the side branch from which it originated. In other segments the channel can be followed till it divides into smaller branches and these eventually get smaller till they end in capillaries, as was seen in the serial sections of the segments shown in figs. 48-53 and 72-74. More frequently, the channels that originated from two different side branches in the same segment, joined together, thus forming a direct link between the two branches (figs. 61-65, 72-77 and 80-85).

An opportunity to study this matter further, was provided by a series of experiments done by Dr. G. M. Wilson and myself, on the collateral circulation and the causes of gangrene in the lower/

lower limbs. The vessels, while still in situ, were injected with lead phosphate suspension, coloured by Trypan blue (see Schlesinger, 1938). An X-ray photograph of the limb was taken and then the main vessels and their branches were dissected out, dehydrated in alcohol and put in clearing solutions (see Winternitz et al 1938). The following points in relation to this part of the work were found:-

1. The X-ray photograph showed that large stretches of the affected vessels were not filled with the opaque substance. The collateral circulation was well shown.

2. After the vessel walls were rendered transparent, the same appearance was found. Segments that did not show any coloured lead phosphate in them were sectioned and examined microscopically. The lumen was found to be completely obliterated.

3. Practically all the side branches coming off the occluded segments were filled with the injected material. The only possible passage of the injected material was through the collateral circulation since these side branches had no direct connection with the patent parts of the main lumen which were filled with the coloured lead phosphate.

4. Recanalisation of the occluded main lumen was taking place. The channels originated from the side branches and ran through the occluded lumen for some distance. They either ended blindly or were connected with other canalising channels coming from other side/

side branches (fig. 91).

5. There was no connection between these canalising channels and the patent main lumen.

6. The occluded segments that had no side branches did not show any of these canalising channels.

The functions of these channels appeared to be:-

1. They helped to supply the vessel walls and the connective tissue in the lumen with arterial blood, while the venous return was carried back by some of the sinuses as described above. This does not mean that the lumen is not supplied by arterial blood by the capillaries from the vasa vasorum, but the big non-elastic-coated sinuses were connected with the venous side of the circulation. (N.B. for a full discussion on the origin of the vasa vasorum and the arterial and venous supply to the vessel walls see Ramsey, 1936 and Winternitz et al 1938).

2. The channels formed a link or shunt between two patent, functioning side branches. They appear to act as an anastamotic link between two different parts of the collateral circulation.

In the vessel segments occluded by arteriosclerotic plaques, the canalising channels acted as links between the side branches and also helped to supply the nutrition to the surrounding tissues as evidenced by the relative preservation of adjacent parts of the arterial/

arterial wall. (see also Leary, 1934, Paterson, 1936).

As shown by the microscopical examination of occluded segments, many of the elastic-coated channels were affected later by the same occlusive process that occurred in the main vessel. They became thrombosed and organised and as shown in figs. 50-53 and 61-64, some of them became recanalised by elastic-coated channels originating in the patent side branches. When the side branches were themselves occluded, at the same time, no such recanalisation took place (figs. 61 and 74).

After having shown how the elastic-coated channels were invariably connected directly with the general arterial system, it becomes easy to answer the question as to why the elastic tissue developed around them. It has been shown that every functioning arterial lumen whose size allows the bllod to flow through it under some pressure, acquired a limiting elastic coat outside the endothelial lining (Gery et al 1939, Leriche, 1946). The number of elastic fibres and the thickness of such a coat depended upon the pressure of the blood flowing inside it and the age of the channel. This also explains why elastic fibres were found amongst the layers of the sclerosed plaques since these developed in successive stages while the lumen was still functioning.

Sources of the new elastic fibres: The theories about the elastic tissue formation in the adult organism are the same as those discussed under the formation in the embryo (see Chapter II). In addition to these, some others suggested that the new elastic fibres were a re-arrangement of the pre-existing ones present/

present in the surrounding tissues (Milne, 1908 and J. W. Dawson). Regarding the source of the new elastic fibres around the canalising channels, Jores (1900) contended that the canalising channels developed from the angioblasts growing in from the vasa vasorum and that the elastic tissue surrounding them was a prolongation from the elastica of the media. Buerger could not find any connection between the newly formed elastica and that of the main vessel wall. He suggested that they developed from collagen fibres under the influence of the blood flow. Borchardt, (1926) thought that they were formed by the proliferating endothelial cells lining the main vessel lumen. Krompecher (1930) was of the opinion that they developed from elastoblasts which migrated from the original intima into the thrombus with the ingrowing vasa.

It was not possible to ascertain the time of the first appearance of the elastic tissue around these channels since they were already well developed when the limbs were amputated. It was nevertheless possible to study the stages of their development in some of the segments.

The new elastic fibres developed first around the part of the channels nearest the side branches. They then gradually formed along the channel wall as it penetrated deeper into the main lumen. The deeper parts were surrounded by fewer elastic fibres than the parts nearest the side branches. The new elastic tissue was first seen forming extra-cellularly around some of the connective/

connective tissue cells that were outside the endothelial lining of the channels (figs. 92 and 93). These cells did not differ morphologically from the other connective tissue cells in the intima or lumen. The first elastic elements were in the form of granules and small fibrils which eventually fused together to form elastic fibres. They were also argyrophilic in the early stages of their development. The further development of these elastic fibres and the formation of the elastic coats was very similar to the process described in the chick embryo.

It appears that when these channels begin to carry arterial blood, flowing under some pressure, inside them, this causes the elastic producing cells which are normally present in the intima of the vessel walls to proliferate and they are stimulated to start functioning again. These elastic-producing cells, starting from the intima of the side branches, spread along the walls of the canalising channels outside their endothelial lining (see fig. 92). The amount of elastic tissue formed is proportional to the importance of the channel and its age. This process of new elastic tissue formation is a good example of how the physiological needs of the body can stimulate and influence the specialised cells in the body to start functioning again and lay down their products. Many of the other blood sinuses in the occluded lumen were even wider and bigger than the elastic-coated channels but no elastic tissue developed around them since they were not connected directly with the arterial circulation.

Summary:/

Summary:

1. The development of the elastic-coated channels in occluded vessel lumina was described.

2. The elastic tissue around them appeared to develop in the same way it did in the embryo.

3. The influence of the physiological needs of the adult organism on the development of elastic tissue was stressed.

APPENDIX TO CHAPTER IV.

CASE I.

Alexander W. (ref. no. M.H.A. 298).

Age: 40 years.

Sex: male.

Duration of illness: 4 years.

Present illness: coldness of both feet; absence of pulse in both lower extremities. Progressive gangrene in the left leg. Supra-condylar amputation 30: 7: 42.

Pathological report:

All parts of the arterial tree of the leg show similar changes varying only in degree. There is obliteration of all the main arteries by vascularised connective tissue. Many of the new capillaries in the lumen are continuous with those in the media and can be traced right into the vasa vasorum, in the adventitia. The lumen is full of haemosiderin granules. The intima is thickened and the internal elastic lamina is proliferated, in some parts fragmented and in other parts there are calcium deposits around it. The media shows moderate fibrosis and marked vascularisation. The adventitia is extensively fibrosed and shows foci of round-cell infiltration. The veins show varying degrees of intimal fibrosis and one shows a small recently organised mural thrombus.

Diagnosis: Thrombo-angiitis obliterans.

Description/

Description of a segment from the middle part of the posterior tibial artery:-

Fig. 39: The lumen is occluded by an old organised thrombus. It contains haemosiderin pigment deposits and several large vascular spaces. Two of these spaces are surrounded by elastic coats (a and b). A branch of the vessel is only partially occluded by the same kind of tissue and the remainder of the lumen is surrounded by an elastic lamina.

Fig. 40: The same branch is seen coming off the main vessel. Note the big sinus entering the media at the site of junction. The channel - a - is now nearer the side branch.

Fig. 41: The lumina of the channel - a - and of the side branch are seen communicating, and the elastica that surrounded both of them is now continuous.

Fig. 42: The side branch is separating from the main vessel. Note the increased elastic network of the intima at the place of junction (elastic cushions).

Fig. 43: The continuation of the channel - a - is now smaller in size and is surrounded outside its elastic coat by dense acellular fibrous tissue.

Fig. 44: Further down, the channel - a - ends blindly. Another side branch is seen in the lower right-hand corner. It later joins with the channel - b - in the same manner as that described above.

Figs. 45 & 46: Show how the capillaries and sinuses which traversed the occluded lumen, media and adventitia, can be followed to their termination in one of the venae commitantes accompanying the artery.

Fig. 47: A complete reconstruction-diagram of the vessel segment described above.

Fig. 39. (x 25).

Fig. 40. (x 25).

Fig. 41. (x 25).

Fig. 42. (x 25).

Fig. 43. (x 25).

Fig. 44. (x 25).

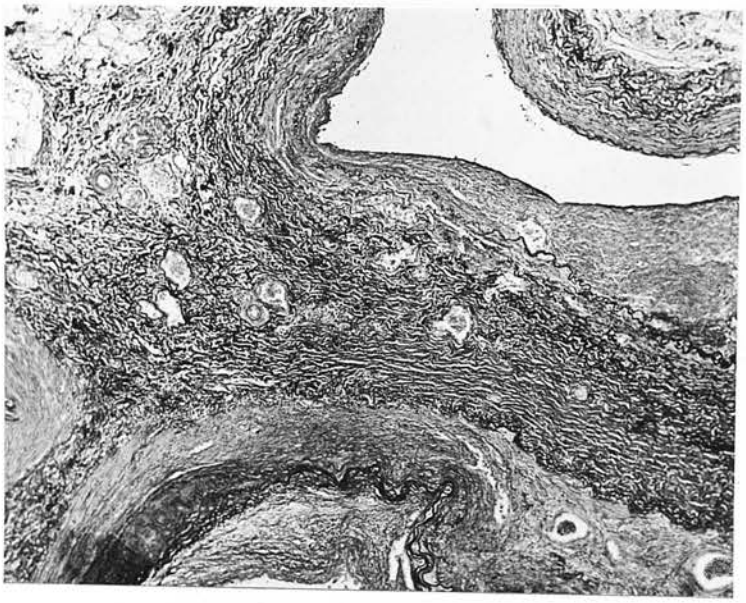

Fig. 45. (x 80).

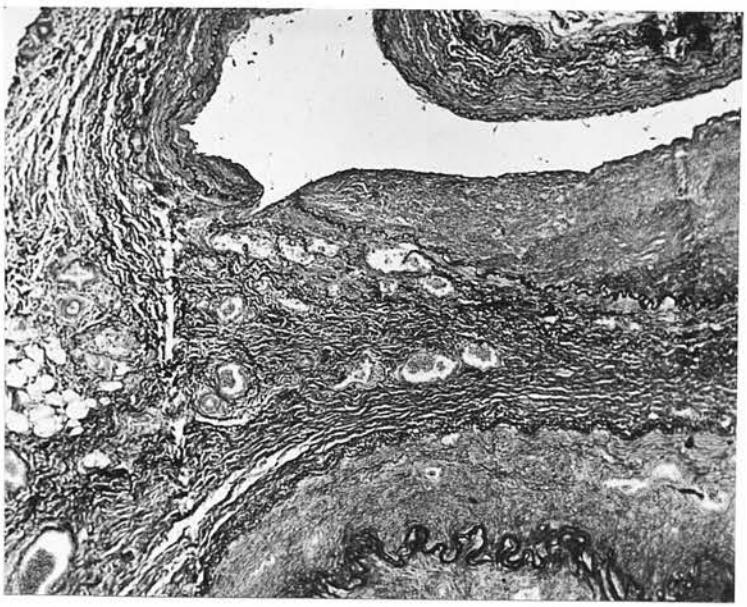

Fig. 46. (x 80).

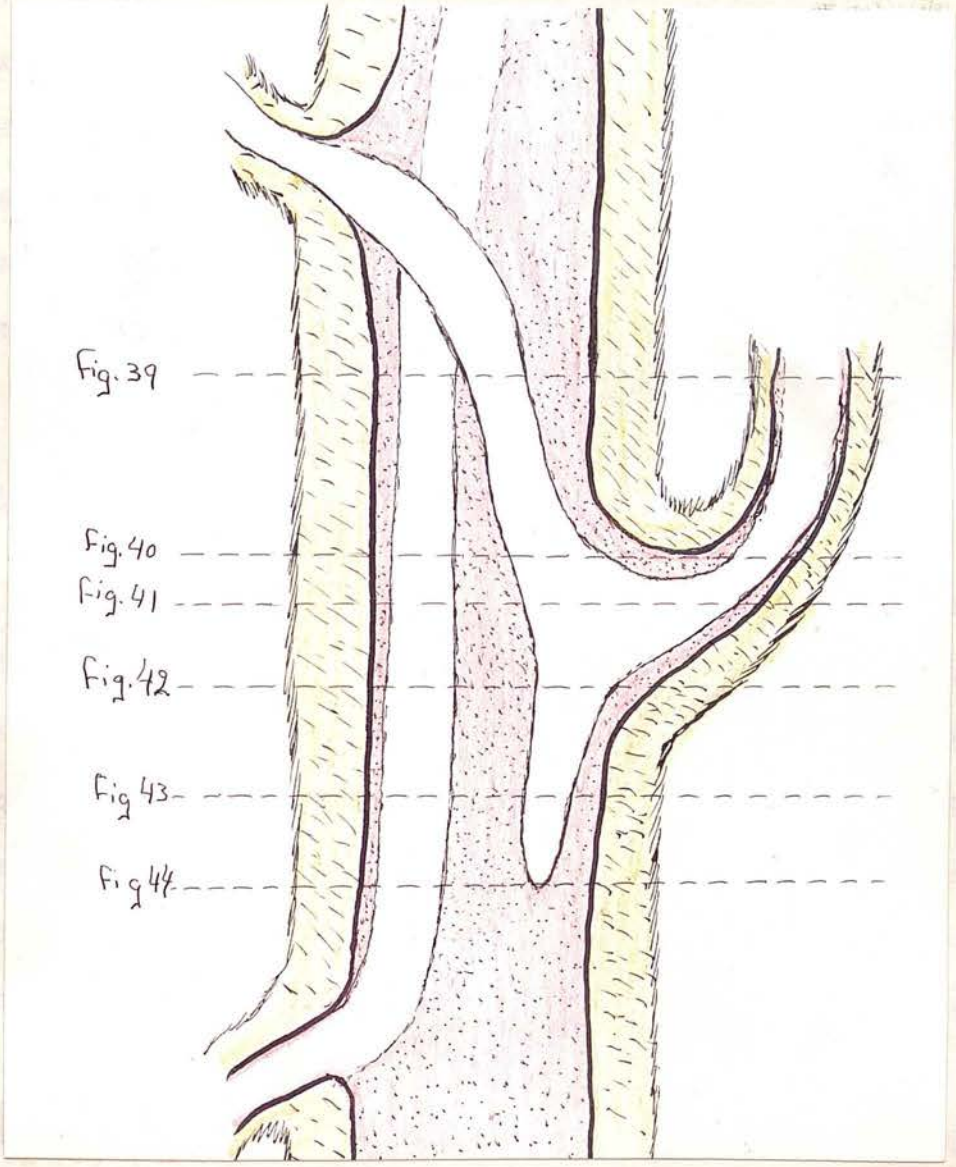

Fig. 47.

Fred D. (ref. no. M.H.A. 1069).

Age: 35.

Sex: male.

Duration of illness: 5 months.

Present illness: started as intermittent claudication in the right leg. Both feet were persistently cold. Four months ago discolouration of the right big toe began, progressing to gangrene. There was no response to any conservative treatment. Amputation on 24:5:44.

Pathological report:

The anterior tibial artery is the only main vessel seriously affected. Its lower end is completely occluded by an old organised thrombus. There are scattered foci of round cell infiltration in all the vessel coats and lumen. The internal elastic lamina is reduplicated but otherwise intact. The media shows moderate fibrosis and vascularisation. The adventitia shows an increase of fibrous tissue. The accompanying veins are relatively normal.

The posterior tibial vessels are normal along their whole length.

Diagnosis: Thrombo-angiitis obliterans.

Description of a vessel segment taken from the lower end of the anterior tibial artery:-

Fig. 48: The lumen is filled with vascular connective tissue, foci of round cells/

cells and haemosiderin pigment. It contains several channels lined by endothelial cells, and many of these are seen penetrating to the media. Several other channels are surrounded by elastic coats. The biggest of these latter channels is filled by more recent vascular connective tissue. The internal elastic lamina is thickened, the media is extensively vascularised and infiltrated with round cells. The adventitia show moderate fibrosis.

Figs. 49 & 50: The capillaries and endothelial-lined channels eventually enter the media or join one of the elastic-coated channels. These join together into one large channel which is now surrounded by several layers of elastic fibres. A side branch is starting to come off the main vessel.

Fig. 51: The division is more obvious now. Notice the increase in the number of elastic fibres in the intima of the vessel and around the central channel which has gradually grown larger in diameter.

Fig. 52: The central channel has grown so large that its elastic coats have merged with those of the intima of the main vessel. The lumen of the central channel has joined that of the side branch. The innermost elastic layer of the central channel is continuous with the internal elastic lamina of the side branch.

Fig. 53: Further down, after the separation of the side branch, the lumen of the main vessel (on the left) is seen much reduced by the fibro-elastic proliferation of the intima. The rest of the lumen is filled with young vascular connective tissue replacing a thrombus.

The intima of the side branch (on the right) show similar changes

to/

to those affecting that of the main vessel, but the lumen is fully patent. Most of the vascular spaces in the central channel, described inside the main vessel, collect in that functioning lumen of the side branch.

Fig. 54: A reconstruction-diagram of the vessel segment described above.

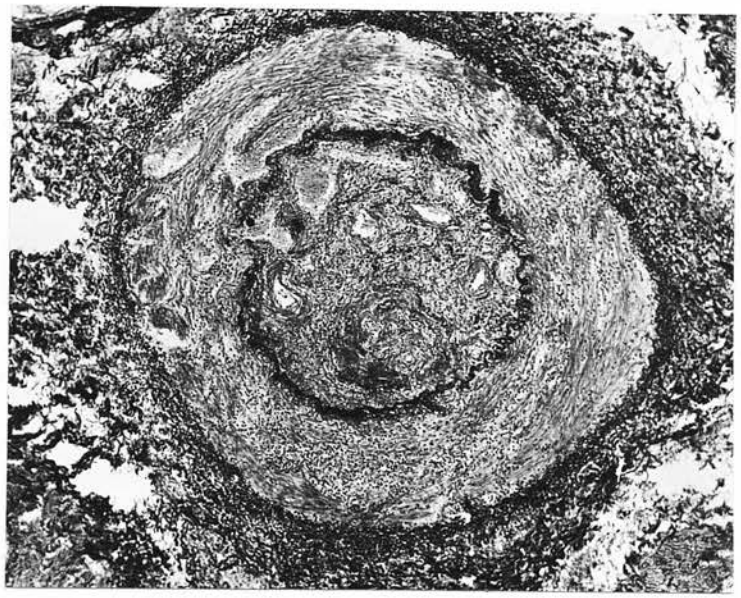

Fig. 48. (x 60).

Fig. 49. (x 60).

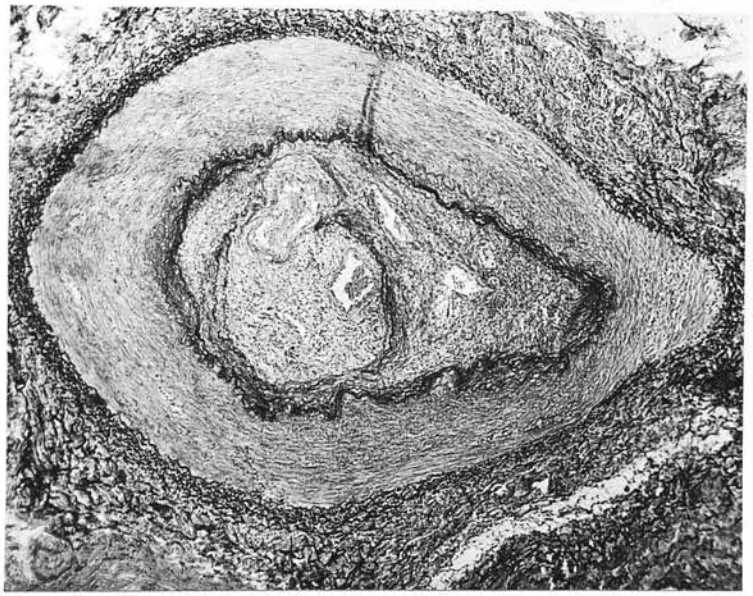

Fig. 50. (x 60).

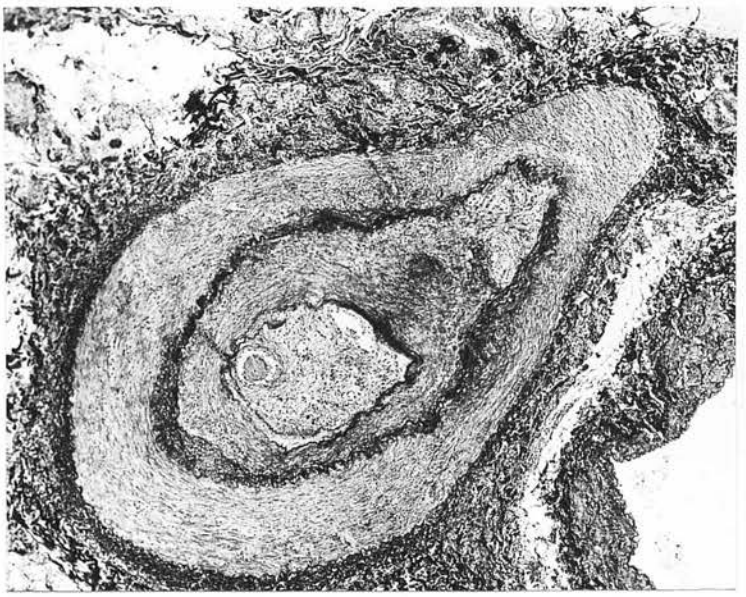

Fig. 51. (x 60).

Fig. 52. (x 60).

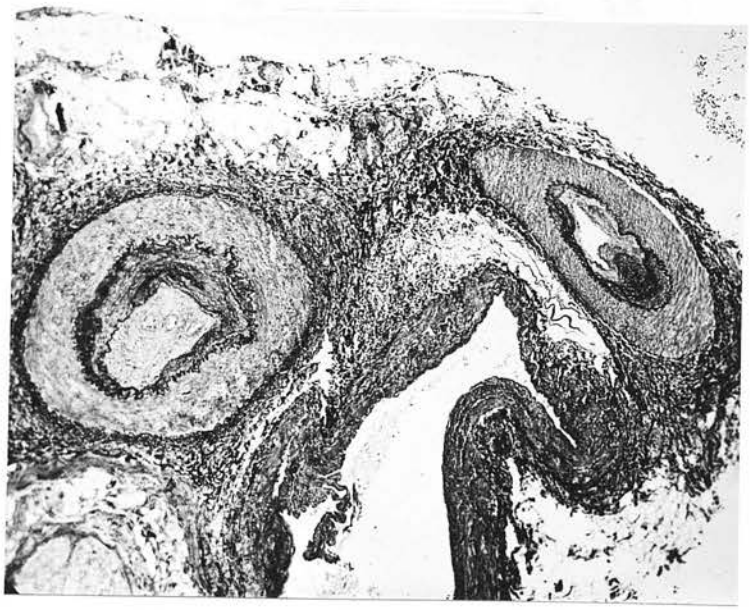

Fig. 53. (x 40).

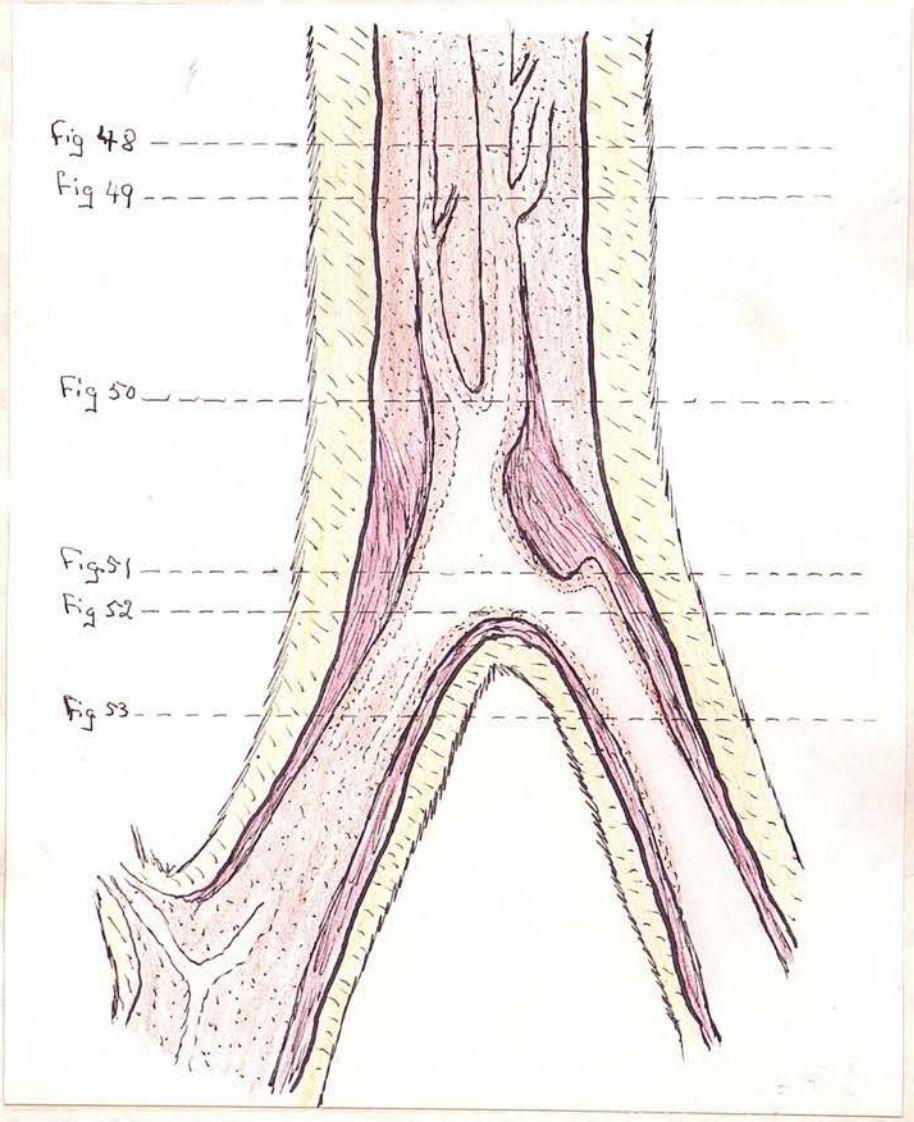

Fig. 54.

James M. (ref. no. M.H.A. 973).

Age: 31.

Sex: male.

Duration of illness: 5 years.

Present illness; started when joining the army in 11:11:39. He felt numbness of right toes and pain in the right calf after a 5 - 6 miles march. Both were relieved by rest. In September, 1943, he had intermittent claudication after walking a distance of about 300 yards and the right leg became swollen and discoloured 2:11:43 : injection of 2 c.c.s. Novocaine into the external peroneal nerve 18:11:43: proctocaine injection into posterior tibial and external popliteal nerves 28:1:44: Cordotomy 6:3:44: Amputation of right leg below the knee.

Pathological report:

The lower ends of both tibial arteries were affected. Both arteries show similar changes of old thrombosis and organisation, varying only in degree. Some segments show canalising channels. The internal elastic lamina is proliferated in some regions and deficient in others. There is a varying amount of fibrosis, vascularisation and round-cell infiltration of the media. The adventitia show a varying degree of perivascular fibrosis. The veins show only slight medial fibrosis and round-cell infiltration.

Diagnosis: Thrombo-angiitis obliterans.

Description/

Description of a vessel segment taken from lower part of the anterior tibial artery:-

Fig. 55: The original internal elastic lamina is absent at the points where capillaries traverse it. A crescent-shaped area is seen on the right-hand side of the lumen. It is limited externally by the internal elastic lamina and internally by a new thick elastic coat. It is made of old granulation tissue. A small endothelial-lined sinus is seen penetrating into the media. A small elastic-coated channel is seen near its lower edge. The rest of the original lumen is partially filled by young fibrous tissue replacing a thrombus, thus leaving only an eccentric patent blood channel which is lined by a thick elastic lamina.

Fig. 56: The small elastic-coated channel is seen communicating with the patent big channel.

Fig. 57: The section shows the junction of a completely patent side branch with the main vessel. The elastic coat surrounding the functioning blood channel is continuous with the internal elastic coat of the side branch.

Fig. 58: Another side branch is seen coming off the main vessel on the opposite side of the vessel. Its lumen communicates with the inner area of young fibrous tissue by means of an elastic-coated channel.

Fig. 59: At the lower end of the vessel segment, the two elastic-coated channels whose communications were described above, are seen.

Fig. 60: A reconstruction diagram of the vessel segment described above.

It/

It is most likely that the vessel was affected in two stages. First a mural thrombus formed and became completely organised and the remaining functioning lumen became lined by an elastic coat (see text). Later another similar event occurred.

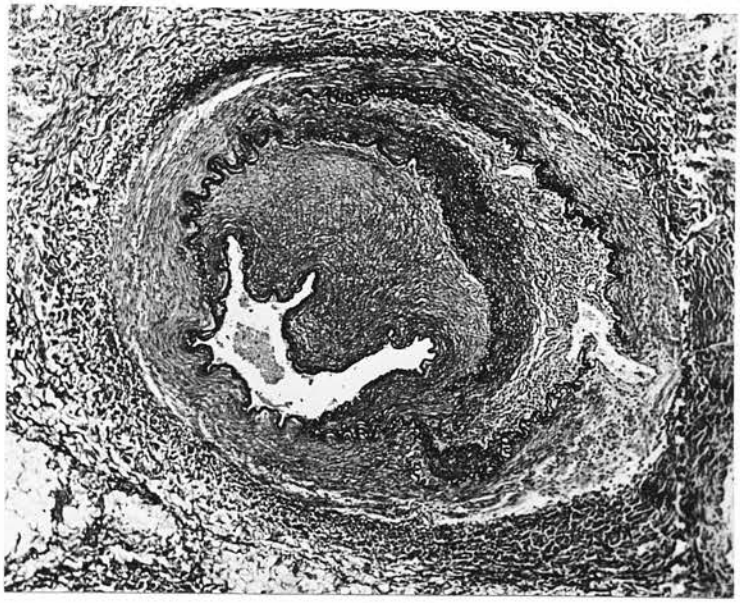

Fig. 55. (x 60).

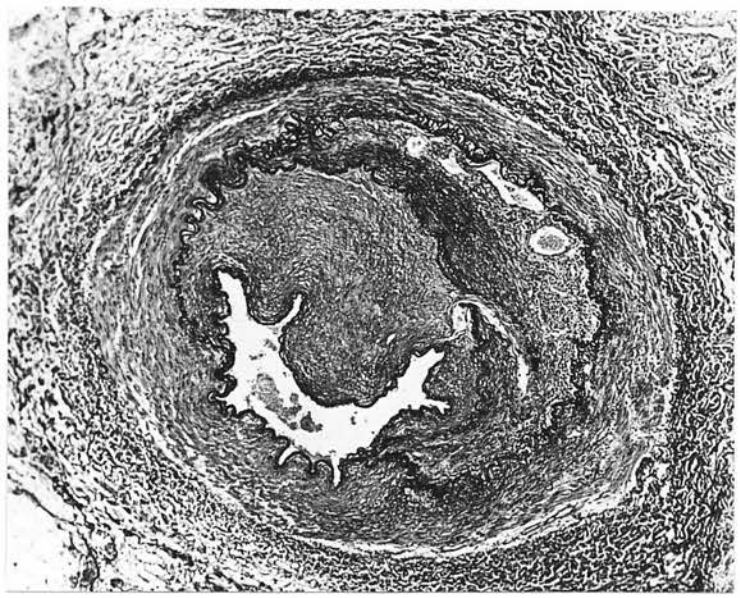

Fig. 56. (x 60).

Fig. 57. (x 40).

Fig. 58 (x 40).

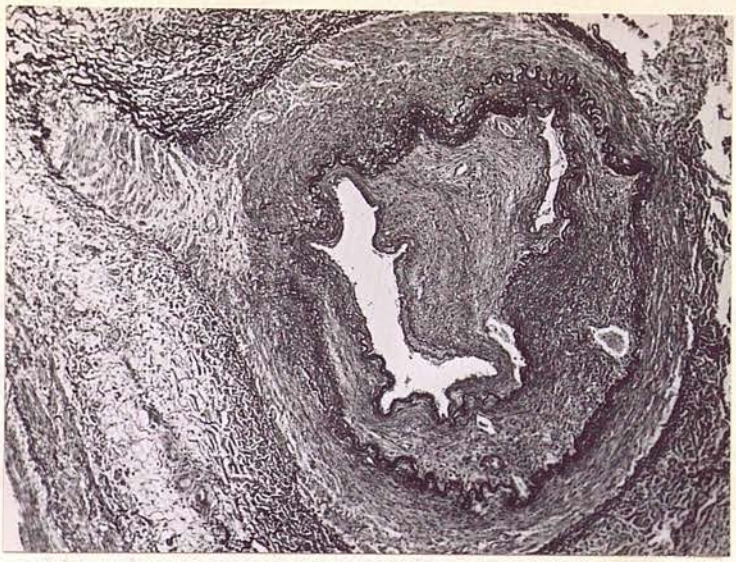

Fig. 59. (x 60).

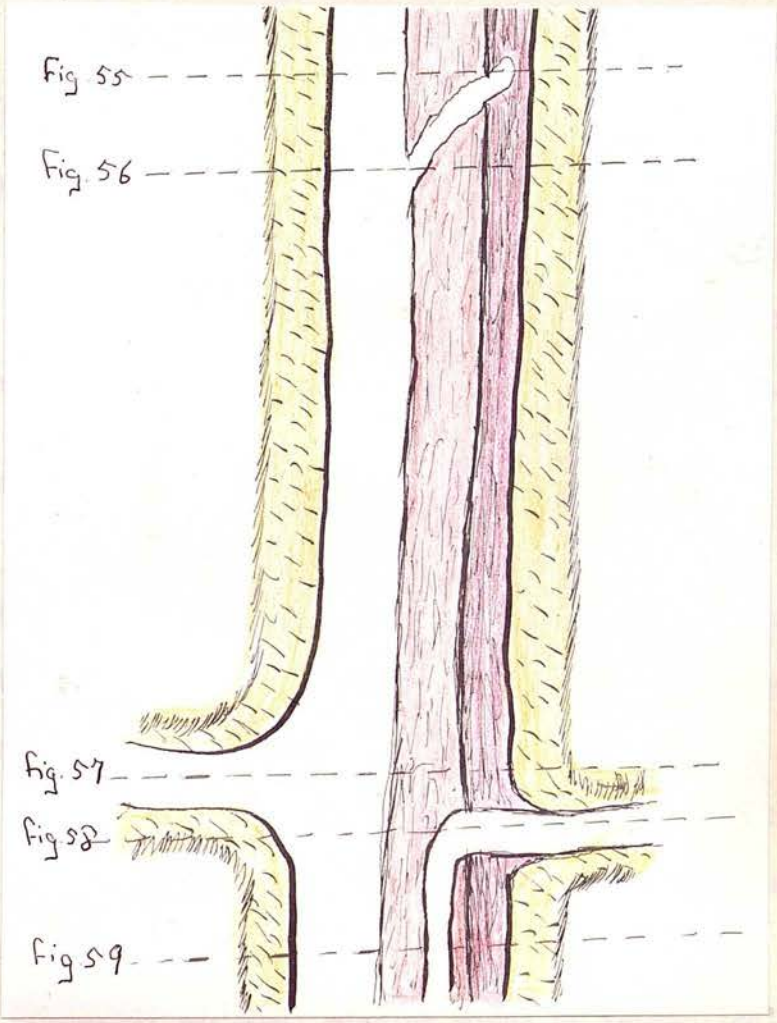

Fig. 60.

Andrew H. (from Ward 7, R.I.E.).

Age: 40.

Sex: male.

Duration of illness: 3 years.

Present illness: amputation of fourth toe two years ago. Amputation of leg 6:12:46.

Pathological report:

The popliteal, posterior tibial and upper two-thirds of the anterior tibial arteries were patent and only showed varying degrees of intimal thickening.

The lower third of the anterior tibial artery was occluded by a recent thrombus. Organisation was just commencing at the periphery.

The dorsalis pedis and plantar arteries were occluded by more advanced organised thrombi. They showed some recanalised channels which were occluded by a more recent organised thrombus.

Diagnosis: Thrombo-angiitis obliterans.

A segment of the dorsalis pedis was examined in serial sections:-

Fig. 61: The original internal elastic lamina is intact but refractory to the Weigert stain. The lumen is partially replaced by a thick layer of fibrous tissue containing numerous small elastic fibres arranged parallel with the lumen. It is limited internally by a thick elastic lamina. The rest of the lumen is filled with a recently organised thrombus showing signs of recanalisation. This thrombosed/

thrombosed lumen is continuous with that of a side branch which is occluded by the same kind of young connective tissue. The media is vascularised and greatly thickened. The adventitia shows extensive fibrosis binding the adjacent vessels and nerves together.

Fig. 62: Shows the condition of the vessel just after the side branch is given off. Note the small recanalising channel in the centre.

Figs. 63-65: Another side branch is being given off. The central area that is filled with the recently organised thrombus is seen communicating with the lumen of the side branch. This side branch has been affected by a similar process as the main vessel but its lumen is only partially occluded by a recent thrombus.

Fig. 66: Lower down the fibro-elastic layer is now thinner while the rest of the lumen is completely occluded by a recently organised thrombus.

Fig. 67: A reconstruction diagram of the vessel segment described above.

N.B. Whether the thick fibro-elastic coat is due to intimal proliferation or is the remains of a very old organising mural thrombus is difficult to say (see text).

Fig. 61. (x 40).

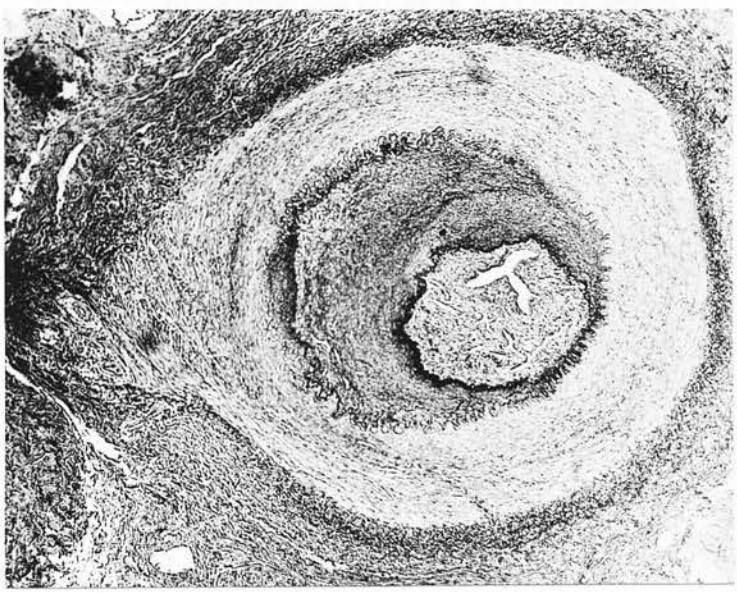

Fig. 62. (x 40).

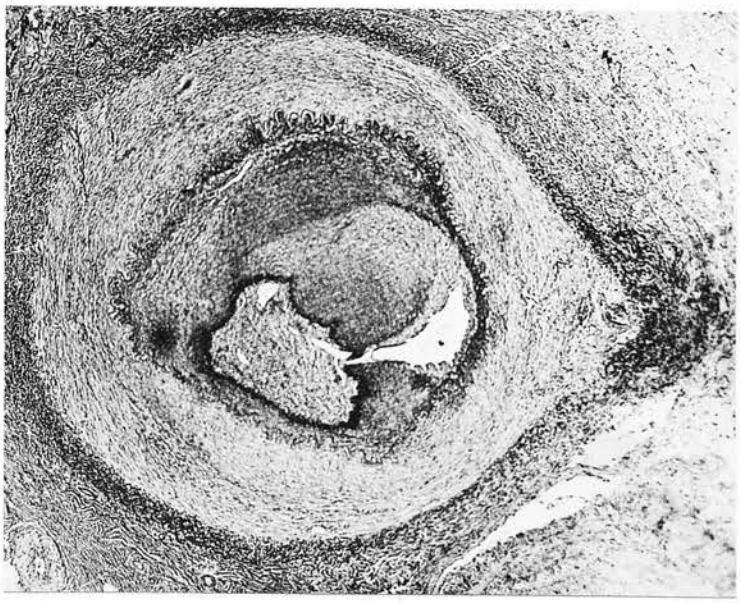

Fig. 63. (x 40).

Fig. 64. (x 40).

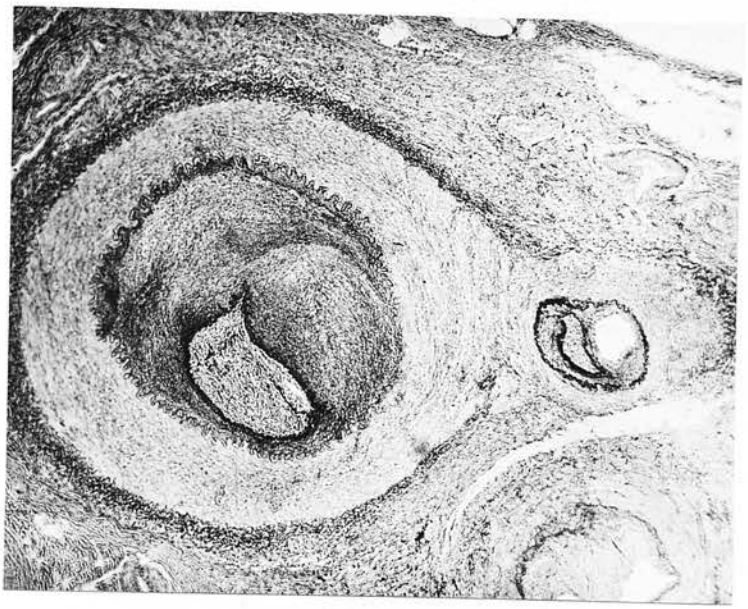

Fig. 65. (x 35).

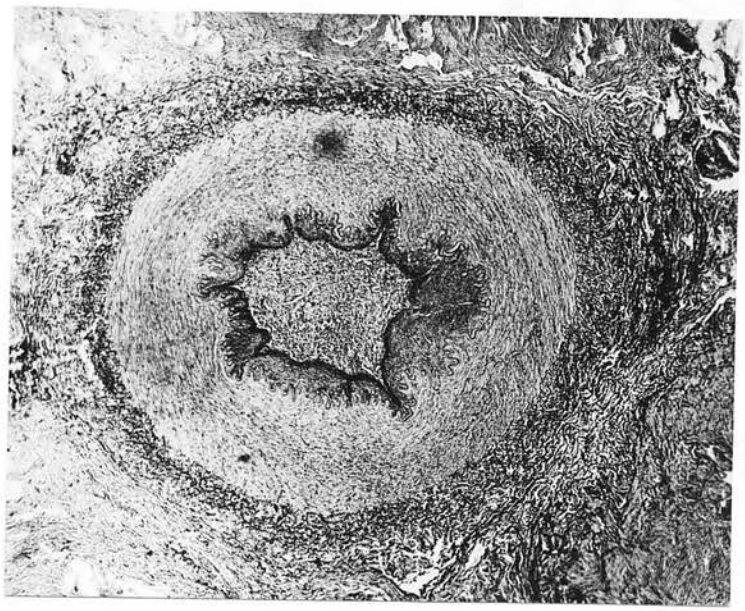

Fig. 66. (x 35).

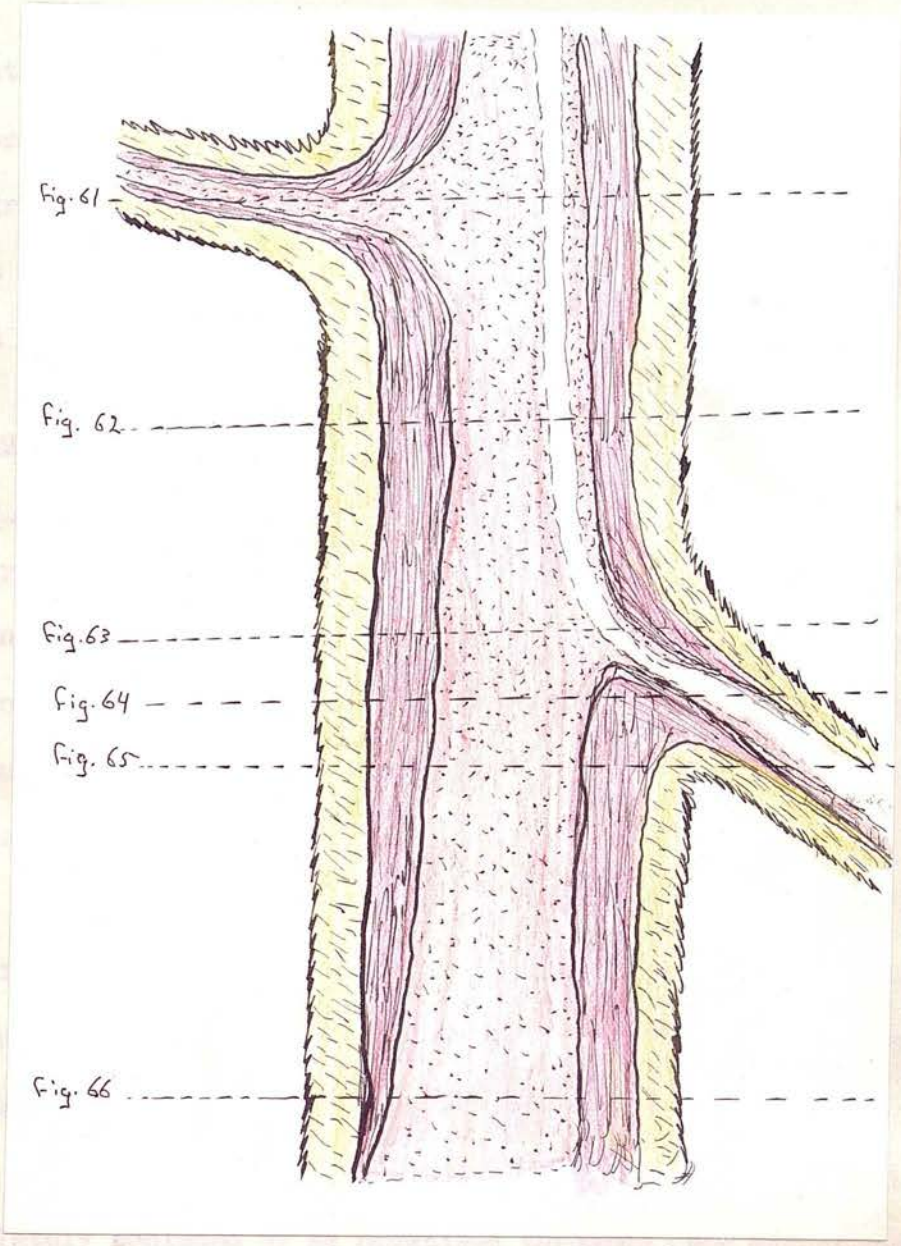

Fig. 67.

Richard F. (ref. nos. M.H.A. 359 and 454).

Age: 50 years.

Sex: male.

Duration of illness: 3 years.

Present illness: November, 1940 - ingrowing toe-nail followed by gangrene of right great toe; gradual improvement. June, 1942 - left great toe involved, gradually becoming worse. Amputation of left leg 17:10:42. Amputation of right leg 23:1:43.

Pathological report:

Left leg: three levels of popliteal vessels; the intima of the artery is thickened in patches and sclerosed. The internal elastic lamina is considerably fragmented and has completely disappeared in some parts. The media is extensively replaced by fibrous tissue. The adventitia is normal. One of the associated veins shows thickening and fibrosis of both the media and intima.

Posterior tibial vessels: the lumen of the artery is patent. The intima shows some thickening with splitting of the elastic lamina. The media shows moderate fibrosis. The veins are moderately fibrosed.

Anterior tibial vessels and dorsalis pedis: the lumen is completely occluded by an organised thrombus (see description).

Right leg: samples were taken from the popliteal, upper, middle and lower stretches of both tibials and from the plantar arteries. The general picture is similar to that described above. All the arteries show varying degrees of arteriosclerosis with elastic splitting,/

splitting, medial fibrosis and patches of calcification. Thrombosis and organisation have occurred in the lower parts of both anterior and posterior tibial arteries.

Diagnosis: Arteriosclerosis with accompanying thrombosis in several segments.

Segments taken from the left dorsalis pedis and lower part of right posterior tibial vessels were serially sectioned and examined.

Left Dorsalis Pedis:

Fig. 68: The lumen is occluded by old acellular fibrous tissue that has replaced a thrombus. Several blood channels are seen traversing it and some of them are covered with elastic coats.
The internal elastic lamina is proliferated; the media is fibrosed, vascularised and shows some foci of calcium deposits. The adventitia shows no special changes.

Figs. 69 & 70: Two side branches are seen coming off the main vessel at opposite poles. Both show thickening of their intimae. All the elastic-coated channels seen in the lumen join up with one or the other. The non-elastic-coated channels penetrate the intima and media to join the vasa vasorum in the adventitia.

Fig. 71: A reconstruction diagram of the vessel segment described above.

Right posterior tibial:

Fig. 72: The lumen is occluded by an old organised thrombus containing some haemosiderin pigment deposits. Several elastic-coated channels are seen traversing it. The internal elastic lamina is proliferated/

proliferated and several foci of calcium deposits are seen in the media outside it. Adventitia is normal in appearance.

Fig. 73: All the elastic-coated channels have collected into one big channel which is partially occluded by a more recently organised thrombus.

A side branch is seen leaving the main vessel; its intima is thickened and its lumen is occluded by an organised thrombus of the same characters as the one described in the elastic-coated channel inside the lumen of the main vessel.

Fig. 74: The big elastic-coated channel is now seen joining the side branch; its elastic coat is continuous with the internal elastica of the side branch. On the right side of the lumen, where another side branch is coming off, the left-hand channel joins another elastic coated channel, occluded also by the same type of organised thrombus.

Fig. 75: The last mentioned side branch on the right side is now better shown. It is occluded and has been recanalised by a big elastic-coated channel which is now seen traversing the occluded lumen of the main vessel. All traces of the big channel described in Figs. 73 and 74 have practically disappeared, since it joined with the blood channel coming from the right-hand side branch.

Fig. 76: A cross-section of the same vessel further down the segment. The elastic-coated channel is partially occluded by a recent thrombus. Some of the non-elastic-coated channels are seen leaving the main lumen to go into the media.

Fig. 77: A fully patent side branch is seen leaving the main vessel. An elastic-coated channel is seen coming from it into the main occluded lumen.

Fig. 78: The main lumen is now recanalised by two elastic-coated channels.

Fig. 79: A reconstructed diagram of the vessel segment described above.

N.B. The blood channels coming from the side branches shown in Figs. 73 and 75 are linked together (see Fig. 74).

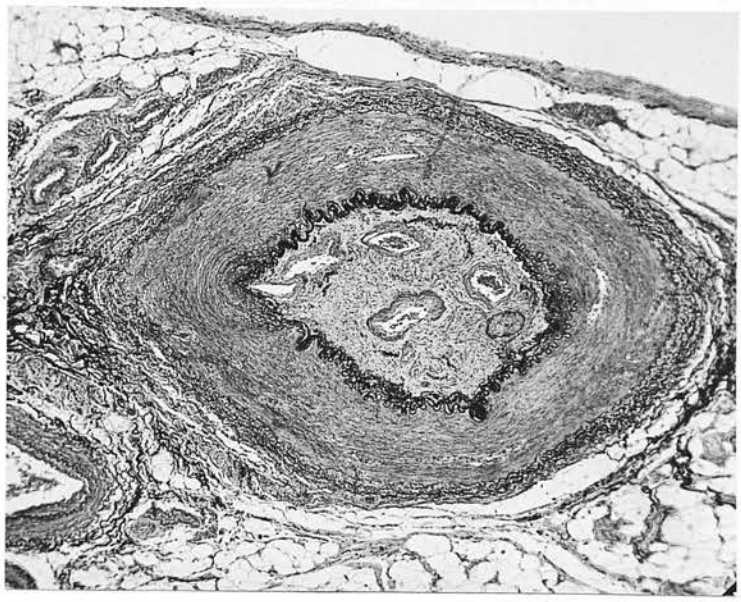

Fig. 68. (x 30).

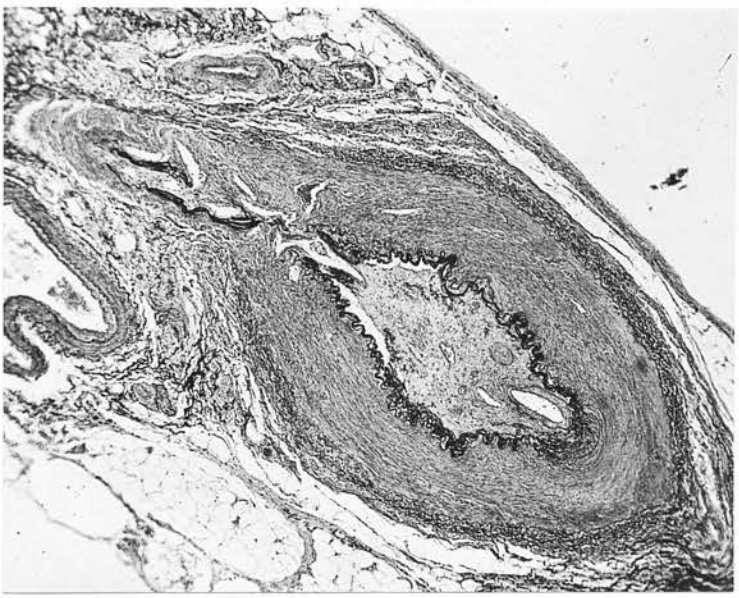

Fig. 69. (x 30).

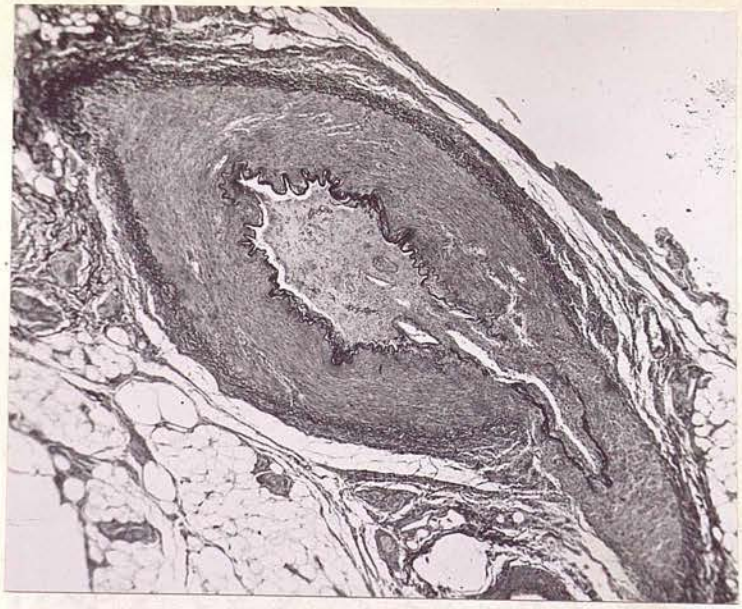

Fig. 70. (x 30).

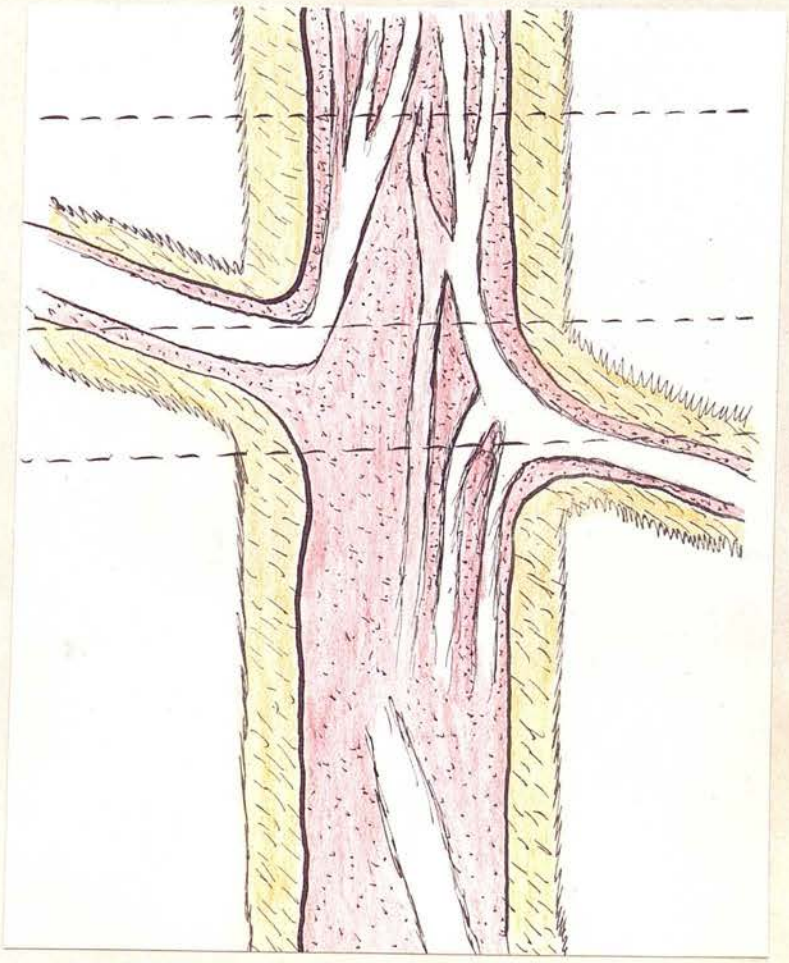

Fig. 71.

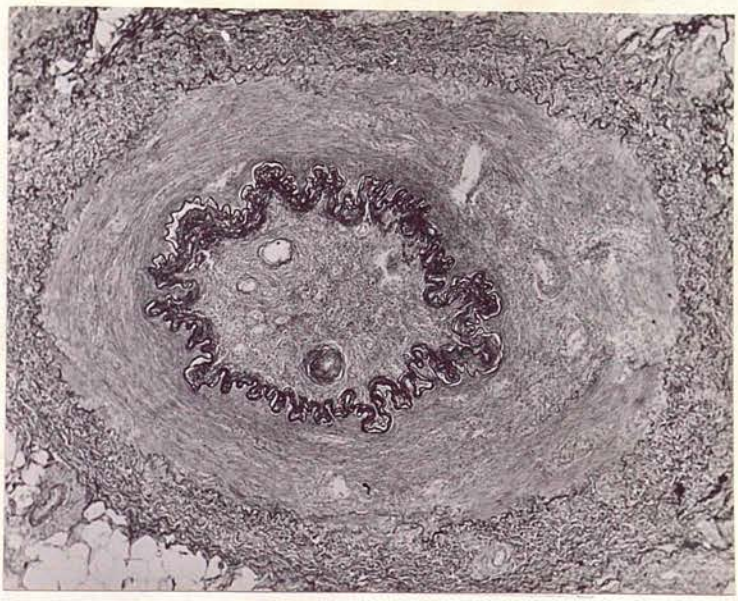

Fig. 72. (x 40).

Fig. 73. (x 30).

Fig. 74. (x 30).

Fig. 75. (x 30).

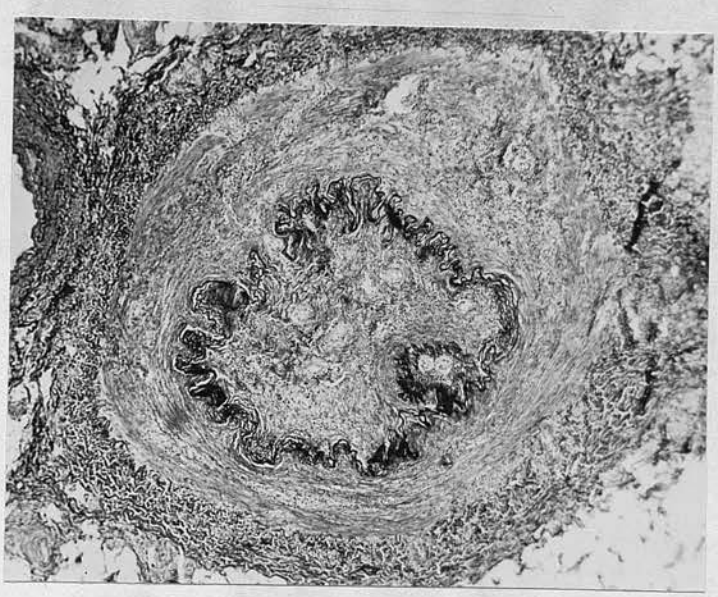

Fig. 76. (x 40).

Fig. 77. (x 35).

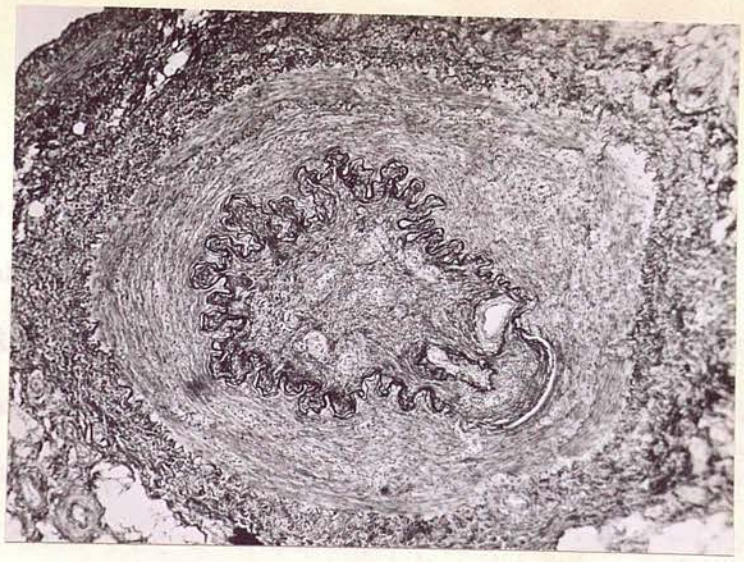

Fig. 78. (x 40).

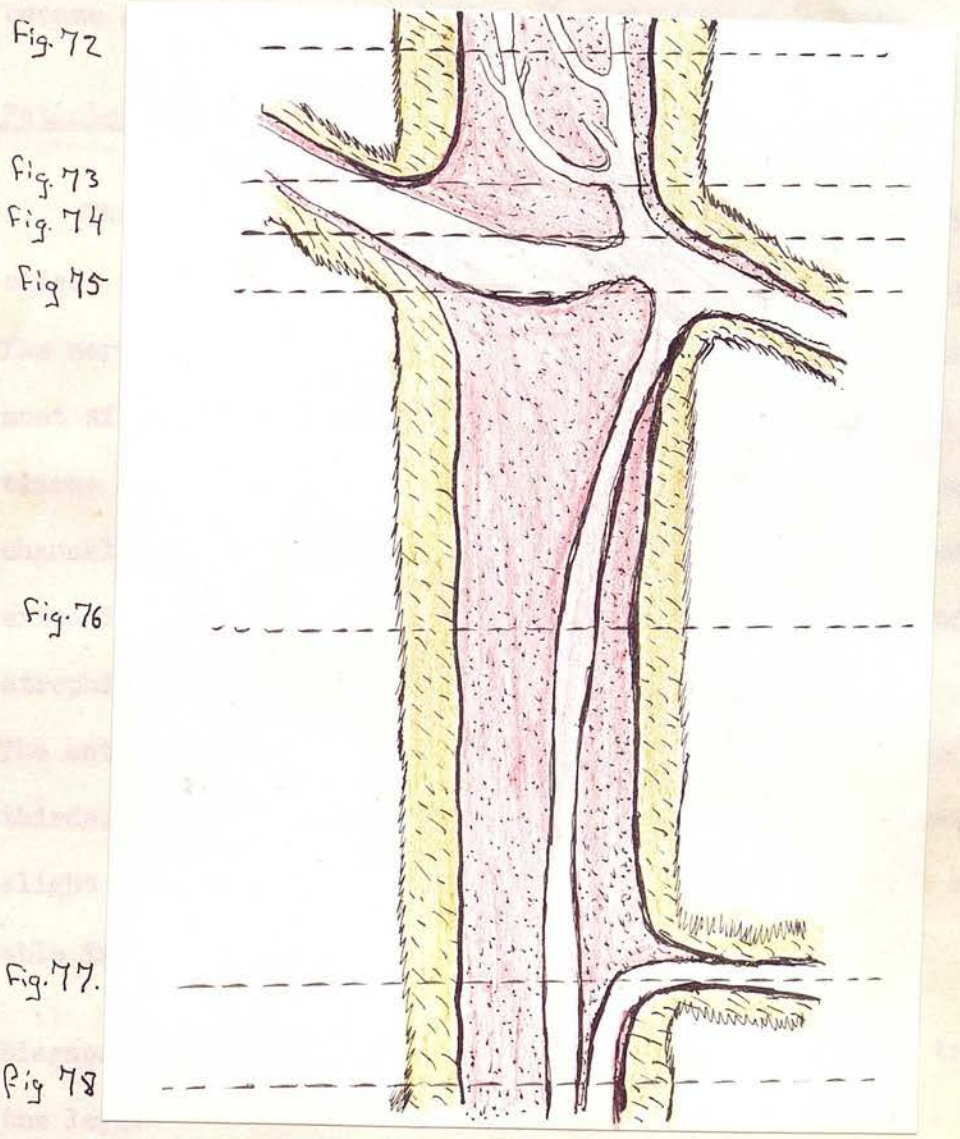

Fig. 79.

CASE VII.

Peter N. (ref. no. M.H.A. 1103).

Age: 55.

Sex: male.

Duration of illness; 5 years.

Present illness: intermittent claudication in the right leg since 1939. Gangrene of the right great toe started in January, 1944. Spontaneous pain since then. Dicoumarin treatment not successful. Petechial haemorrhages on the right third and fourth toes on 24:2:44. Partial amputation of right great toe 24:3:44. Stump became gangrenous. Amputation of right leg on 6:4:44.

Pathological report:

The changes are mainly in the arteries. The veins show slight intimal thickening and medial sclerosis in a few places. The nerves show perineural fibrosis. The posterior tibial is the most affected artery. The lumen is filled by old hyaline sclerosed tissue showing patches of calcium deposits and small recanalised channels. The internal elastic lamina is reduplicated and shows extensive degenerative changes. The media is fibrosed and atrophied. The adventitia shows no significant changes. The anterior tibial artery shows similar changes in its upper two-thirds. At the level of the ankle, it is patent and shows only slight intimal and medial sclerosis. Around it there is considerable infiltration with inflammatory cells.

Diagnosis: old standing atherosclerosis of the arterial tree of the leg.

Segment/

Segment from the lower end of the posterior tibial artery:

Fig. 80: The lumen is filled with hyaline sclerotic tissue arranged in layers and containing many cholesterol clefts. There is a small eccentric blood channel traversing the lumen. It is lined by a thin layer of elastic tissue. The internal elastic lamina of the main vessel is proliferated and degenerated. The media is replaced by fibrous tissue and in some regions is very atrophied and vascularised. The adventitia is relatively normal.

Fig. 81: The canalising channel joins a patent side branch. Its elastic coat is continuous with that of the latter.

Fig. 82: The lumen of the blood channel is larger and is surrounded by many elastic fibres and granules. Note the layering of the main lumen's content. Some traces of elastic tissue can still be seen between these layers. On the left side the vessel bulges with what appears to be the remains of a mural aneurysm (secondary to destruction of the media?). It is separated from the main lumen by a layer of thick elastic fibres.

Fig. 83: This cross-section was taken half an inch below the one shown in Fig. 82. The blood channel has become bigger and the original media adjoining it is better preserved than the rest of this coat.

Fig. 84: The blood channel has joined a healthy patent side branch. The innermost elastic laminae surrounding both lumina are continuous with each other.

Fig. 85: Shows the side branch separating from the main vessel.

Fig. 86: A diagram of the vessel segment described above.

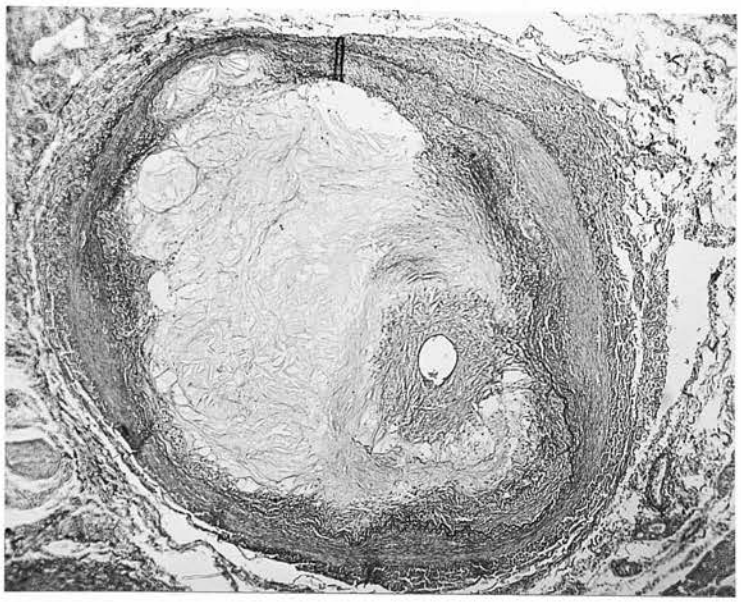

Fig. 80. (x 30).

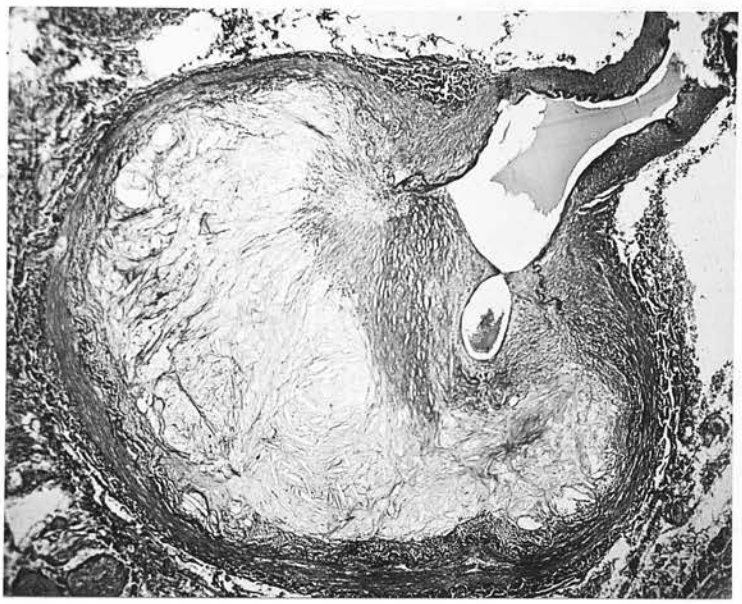

Fig. 81. (x 30).

Fig. 82. (x 30).

Fig. 83. (x 30).

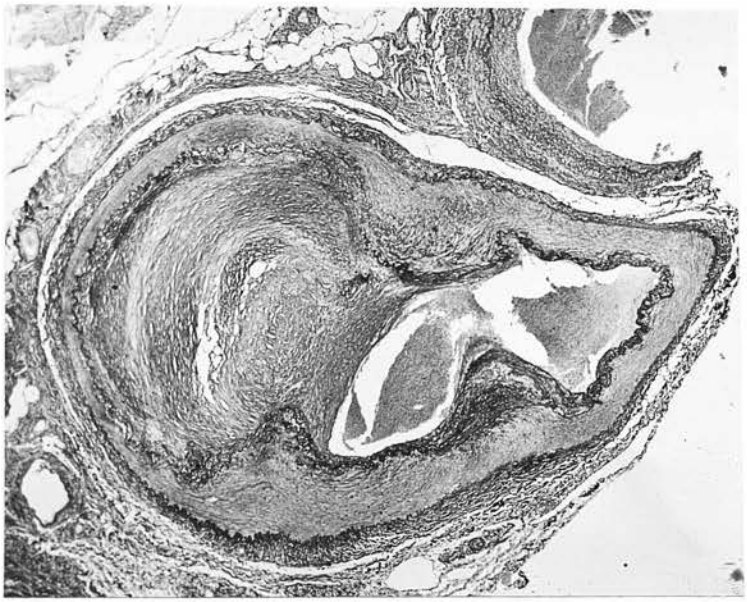

Fig. 84. (x 25).

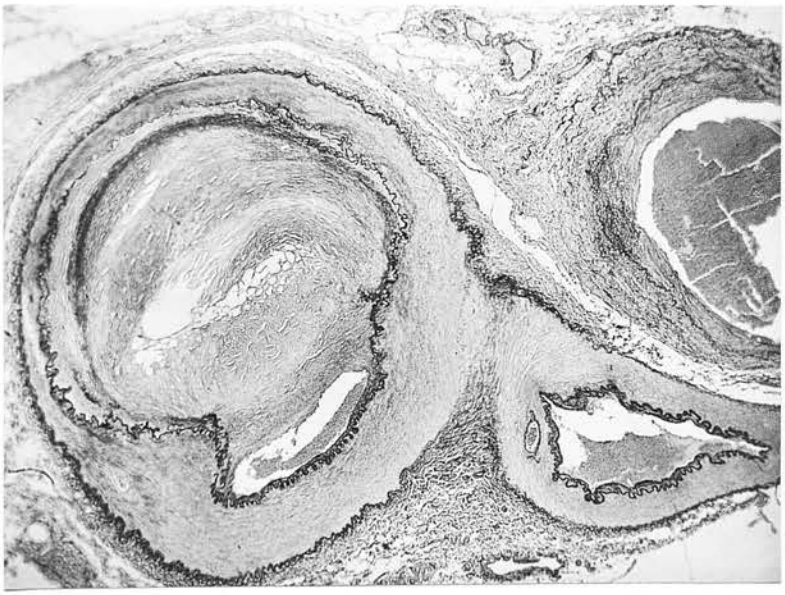

Fig. 85. (x 25).

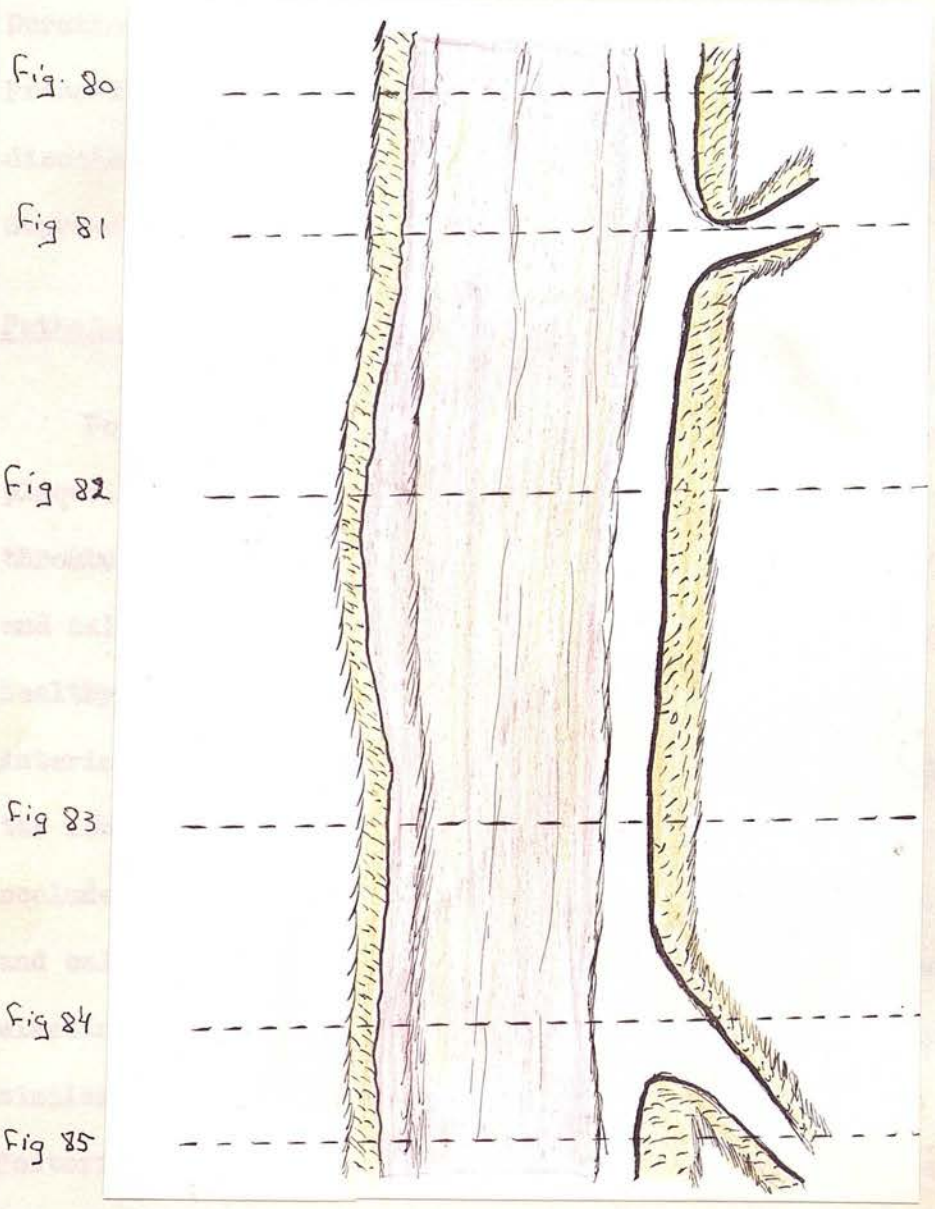

Fig. 86.

CASE VIII.

John S. (ref. no. M.H.A. 470).

Age: 67.

Sex: male.

Duration of illness: 3 years.

Present illness: started with pain in the left foot followed by discolouration of the toes. Fourth toe became gangrenous.

Supra-condylar amputation of left leg 4:3:43.

Pathological report:

Popliteal artery: The lumen is half filled by a sclerotic plaque. The rest of it is occluded by a recent ante mortem thrombus. There is fragmentation of the internal elastic lamina and calcification of the media. The adventitia is relatively healthy.

Anterior tibial artery: The upper stretches are more damaged than the lower parts, otherwise the changes are similar. The lumen is occluded by an old sclerotic plaque. There is extensive fibrosis and calcification of the media. In some parts, the internal elastic lamina is completely destroyed. The plantar vessels show similar changes.

Posterior tibial artery was patent in its upper half but completely occluded in the lower half (vide infra).

Diagnosis: Atherosclerosis with medial degeneration and ante mortem thrombosis of the main vessels of the limb.

A/

A segment from the lower half of the posterior tibial artery:

Fig. 87: The lumen has been completely replaced by an old sclerotic plaque which is made of several layers separated from each other by some elastic fibres. A small elastic-coated blood channel is seen on one side of the lumen. The internal elastic lamina have completely disappeared on one side and the media is replaced by fibrous tissue continuous with the plaque. In other areas many foci of calcium deposits are found.

Fig. 88: The layers of the plaque are better recognised here.

Fig. 89: A side branch is seen coming off the main vessel. It shows the same arteriosclerotic changes but has a part of its lumen patent and is surrounded by an elastic coat. Further down this lumen linked with the canalising channel present in the main vessel.

Fig. 90: A reconstructed diagram of the vessel segment described above.

Fig. 87. (x 40).

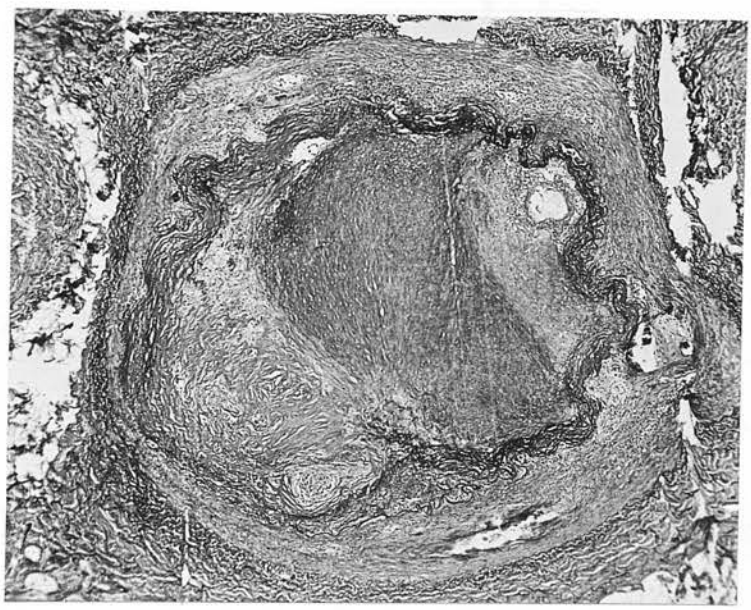

Fig. 88. (x 40).

Fig. 89. (x 30).

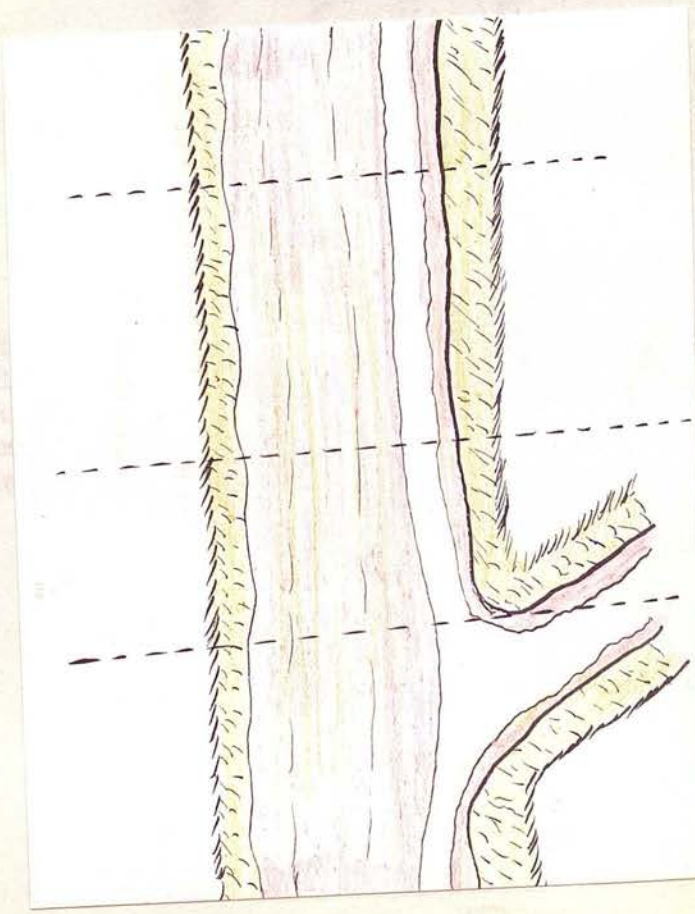

Fig. 90.

Fig. 91.

A photograph of some vessel segments taken after injection and clearing. The channels either ended blindly or were connected to other side branches. The parts not taking the injection materials were proved by microscopical examination to be completely obliterated.

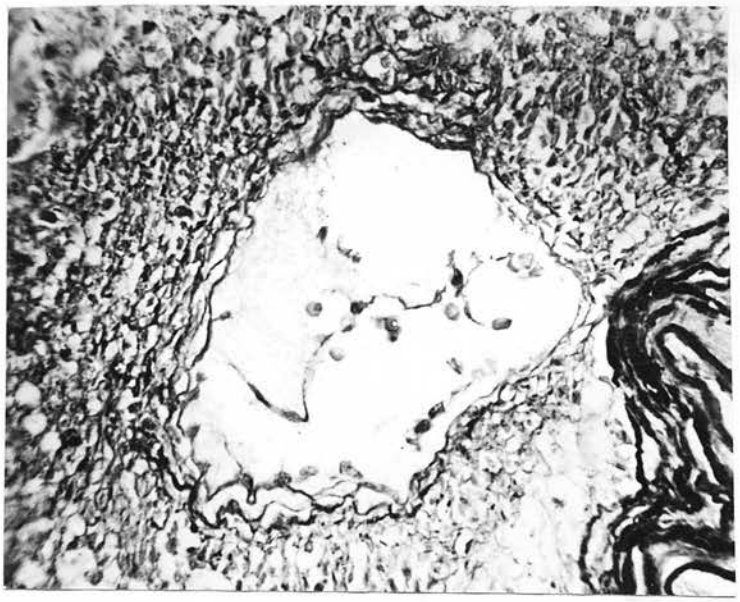

Fig. 92.

Elastic granules and fibres developing extra-cellulary amongst the connective tissue cells surrounding the canalising channels.

Weigert - Haematoxylin - Van Gieson. x 420.

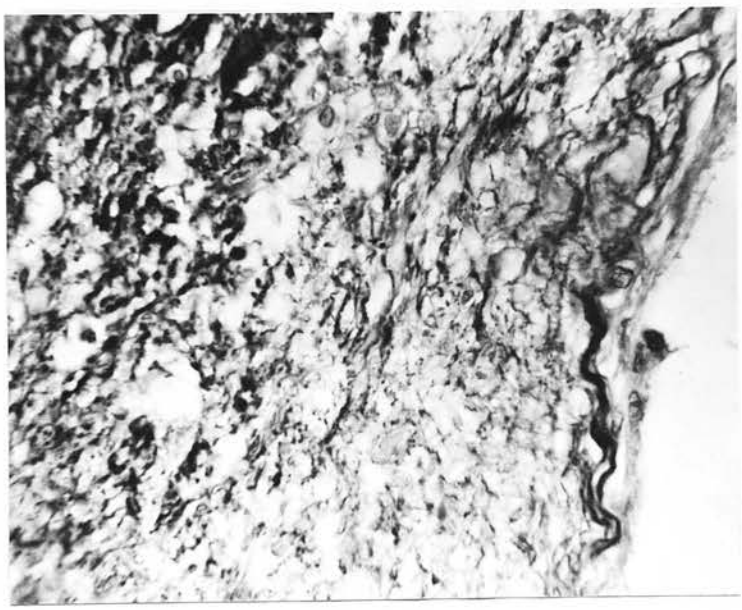

Fig. 93.

Higher power view of part of the channel shown in Fig. 92.

Weigert - Haematoxylin - Van Gieson. x 850.

CHAPTER V.

ELASTIC TISSUE CHANGES IN MAMMARY CARCINOMA.

ELASTIC TISSUE CHANGES IN MAMMARY CARCINOMA.

The effect of neoplastic growths on elastic tissue has been studied by several workers. In 1897, Goldmann (see Willis) noted that it resisted destruction by the invading neoplasms. Since then different suggestions were put forward about its role and behaviour in tumours. Fischer, 1904, and Karrenstein, 1908, believed that certain tumours had the ability to form elastic tissue. Others thought that the elastic tissue seen in tumours was part of the host's tissues (Jores, 1900, Scheel, 1906, Waljaschko, 1907, Willis, 1934, and Bailey, 1940). The latter opinion is the likelier, since none of the metastases of a tumour showing elastic tissue proliferation, contained any elastic fibres.

The various possible reactions of a tissue stroma to tumour growth were classified by Ewing (1940) as:-

1. The stroma may remain passive during the proliferation of the tumour cells.
2. It may be incited to reactive growth.
3. The reactive growth may be carried far enough to reach a neoplastic grade.

The only true elastic neoplasm was reported by Geipel (1906) when he found a pea-sized growth composed of elastic fibres and connective tissue cells. The presence of elastic tissue in mammary carcinomata has been explained by either of the two other possibilities/

possibilities mentioned above. Fischer, Scheel, Waljaschko, Sekiguch (1917) and Cheatle (1923), believed that there was a reactive growth of the elastic tissue around the milk ducts and acini in some of the mammary carcinomata. McConell (1907), Savini and Savini-Castano (1909), Berka (1911) and Reidel (1925) thought that the elastic tissue remained passive, that the elastic hyperplasia was a senile change occurring in normal breasts at the climecteric and could also be seen in breasts affected by hyperplastic cystic disease. They argued that the apparent increase seen in carcinoma was due to the fact that the elastic tissue, being more resistant, remained, while the other tissues were more readily destroyed.

Cheatle (1923 and 1931) studied the elastic tissue changes in the breast in more detail than other workers. He was the first to stress the importance of elastic staining in the study of mammary carcinoma. His work will be referred to several times in this chapter. It is unfortunate that he does not give any figures of the number of mammary tumours which showed elastic changes.

In order to find the relation between the elastic tissue and mammary carcinoma, it was first necessary to study the changes occurring in it in normal breasts with age and in breasts affected by other non-cancerous conditions.

Materials and Methods:

256/

256 biopsy specimens were taken from breast tissues sent to the Pathology Department of the Edinburgh Royal Infirmary during the years 1945-1946. It was possible in some cases to examine only one specimen from each breast. Another 40 specimens of normal breast tissue, representing different ages between birth and 75 years, were taken from autopsy cases.

Two sections of each specimen were stained, one by Haematoxylin-Eosin and the other by Weigert - Haematoxylin - Van Gieson.

Six blocks of mammary carcinomata were serially sectioned and every tenth section was stained by Weigert's elastic stain. A few other representative sections were stained by Haematoxylin-Eosin.

Findings:

1. Elastic tissue changes with age: the mammary tissue of the new-born is composed of several small ducts surrounded by loose connective tissue. Thin strands of elastic fibres are numerous in the skin over the breast but none was found surrounding the ducts. This is contrary to what Cheatle and Cutler (1931) suggested. Up till puberty the development of the mammary ducts follows the general growth of the body. The elastic tissue gradually extends from the corium of the skin and surrounds the termination of the mammary ducts in the nipple. With the onset of puberty, the female breast begins to develop rapidly; the ducts elongate, small epithelial buds grow out of them to form the/

the acini (or ductules) and thus the adult lobular structure is formed. More elastic fibres gradually form along the length of the ducts in 3 - 4 layers, but stop abruptly when they reach the acini (Fig. 94). These ducts are lined by two layers of epithelium; between them and the elastic layer there is a thin layer of sub-epithelial connective tissue. This is continuous with the connective tissue around the acini, which are lined by a single layer of epithelium. Cheatle and Cutler reported that some normal adult breasts had no elastic tissue around their ducts, while in other breasts the elastic tissue extended around the acini. Neither of these findings was present in my series of adult breasts.

It is generally agreed that the cyclic changes in the female have little effect on the elastic tissue development in the breast. In the series under examination it was found that pregnancy and lactation also had little effect on it (Fig. 95).

At the climecteric, there is normally a general atropy of all the glandular elements in the breast. The fate of the elastic tissue around the ducts depends on the build of the woman. In obese women, the breast is composed of a mass of fatty tissue, in which the remaining ducts are scattered. In such ducts the elastica shows no significant changes. In thin women, the whole body of the breast becomes shrunken and atrophied, leaving only some strands of fibrous tissue in which are found the remains of the main mammary ducts. Many of these ducts show an increase of the/

the elastic layers surrounding them (Fig. 96). Any remaining ductules are frequently surrounded by some elastic fibres. Such changes were rarely present in the normal breasts of patients under 50 years.

2. Elastic tissue changes in diseased non-cancerous breasts:
The specimens examined included the following types:-

No.	Type of lesion.	Age of Patients (in years)
57	Hyperplastic cystic mastopathy.	28 - 72
32	Fibro-adenoma.	19 - 45
10	Inflammatory conditions.	26 - 48
6	Duct obstruction.	32 - 46

a. Hyperplastic cystic mastopathy ("chronic mastitis"): the elastic stains differentiated between the cystic spaces of ductal and acinar origin, since the former only were surrounded by elastic fibres. In seven of the specimens, there was an increase in the elastic tissue surrounding the ducts: these ducts were dilated and showed in addition Hyperplasia and desquamation of their lining epithelium (Fig. 97), proliferation of the sub-epithelial fibrous layer (Fig. 98) or both changes together (Fig. 99).

b. Fibro-adenoma: none of the 32 cases showed any significant elastic hyperplasia. It was not possible to demonstrate the role that the elastic tissue around the ducts played in the formation of the intra- and peri-canalisular fibro-adenomata (see Cheatle and Cutler).

c./

c. Inflammatory conditions: these usually occurred in pregnancy or lactation, except for two cases which were tuberculous in origin. In one case of pyogenic infection, the ducts in the affected area showed an increase in both the sub-epithelial and elastic tissues. (Fig. 100).

d. Duct obstruction: the small and medium-sized mammary ducts were dilated and contained inspissated secretions and blood. Some were lined by flattened epithelium, while in others the epithelium formed papillomatous growths. These changes are believed to be due usually to a papilloma growing in one of the main ducts. In two cases there was elastic hyperplasia around some of the small dilated ducts which contained papillomatous proliferation of their lining epithelium. (Fig. 101). A careful search revealed no malignant changes in either breast.

3. Elastic tissue changes in Carcinoma: 151 specimens were examined. In 119 of them there was a marked increase in the elastic tissue around the ducts involved by the cancer process. The specimens were made up of the following histological types:-

Elastic (+)	Elastic (-)	Type
74	17	Scirrhous carcinoma.
25	14	Encephaloid "
17	1	Duct carcinoma.
2	-	Papillary adeno-carcinoma.
1	-	Paget's carcinoma.

These/

These tumours were found in patients of the following age groups:-

Elastic (+)	Elastic (-)	Age groups in years.
8	7	30 - 39
24	7	40 - 49
36	8	50 - 59
43	9	60 - 69
6	1	70 - 79
2	-	80 - over.

In these sections, all traces of the original mammary tissue had usually disappeared, except for the ducts and blood vessels surrounded by elastic tissue. The ducts were present in the centre of the growth and gave a peculiar picture when the elastic tissue around them was increased (Fig. 102). The ducts showing the elastic hyperplasia were the medium and small-sized ones normally present in that area (Figs. 103 and 104). They were surrounded by about 100 - 200 layers of elastic fibres and granules and in many cases this increase could be easily seen by the naked eye. Outside this layer of elastic hyperplasia, the ducts were completely surrounded by the proliferating malignant cells.

The structure of the ducts which showed elastic hyperplasia varied in the same specimen, and in the different specimens examined. Some of them had an apparently normal epithelial lining with empty lumina (Fig. 105). In others, the lumina were narrowed or obliterated by the elastic hyperplasia (Figs.103 and 106).

In/

In still other ducts there was proliferation of the sub-epithelial connective tissue (Fig. 107), while many of the ducts were filled with malignant cells only (Figs. 107 and 108). Several ducts showed a combination of these changes (Fig. 109).

It was rather puzzling to find all these changes in different ducts, in the same specimen, so serial sections of several carcinomatous growths were made. This showed that all the mammary ducts which were surrounded by increased elastic tissue were connected together and met in the centre of the growth. The changes in the ducts were seen to merge gradually into each other at various levels of the same ductal tree. In one particular carcinoma serially sectioned, one of the big ducts in the centre of the growth had several papillomata growing in its lumen (Fig. 110). Further down, the same duct showed a picture of slight epithelial hyperplasia and sub-epithelial connective tissue proliferation (Fig. 111). The changes in the duct at both levels could easily pass as non-malignant, but the surrounding tissues were invaded by tumour cells. This simple epithelial proliferation gradually gave place to cells which completely filled the ducts and whose malignancy was undoubted (Fig. 112). In other branches of the same duct, the lining epithelium was apparently normal, but the proliferated sub-epithelial coat was infiltrated with malignant cells (Fig. 113). The smaller branches of the duct, at the periphery of the tumour, showed similar changes, with gaps in their walls where the malignant cells were spreading outwards (Figs. 114 and 115).

Some/

Some of the small main blood vessels remaining in the tumour area showed elastic hyperplasia in their walls. This was particularly marked in the veins that were completely surrounded by tumour cells (Figs. 116 and 117). These vessels were part of the original mammary tissue. The arteries did not show this change except in their adventitia, but in three cases there was calcification of the internal elastic lamina and the inner layers of the media (Fig. 116). These changes were present only in the area of the breast involved in the malignant growth and not in the unaffected parts.

The one case of Paget's carcinoma showed a marked increase in the elastic tissue of the main ducts in the nipple region. This is in line with the findings of both Sekiguchi (1917) and Cheatle and Cutler (1931).

The increased elastic tissue in all these mammary carcinomata was composed of several layers of small elastic fibres. Sometimes these were collected so close together that it was only with difficulty that one could make out the details of the individual fibres. It was possible in many cases to demonstrate the presence of connective tissue cells amongst these fibres. These connective tissue cells were more prevalent in the elastic layers not infiltrated by tumour cells. They were rarely seen in degenerating elastic layers. The elastic layers gradually became infiltrated by the tumour cells and were broken up into granules

and/

and destroyed (Figs. 104, 105, 106 and 109).

Outside the cancer area, the remaining ducts of the breast did not show any increase in the elastic tissue around them. Of 23 cases of cancerous breasts, which also had hyperplastic cystic mastitis, only three cases showed an increase in the elastic tissue surrounding the ducts in the non-cancerous areas.

A more significant finding was the fact that when the tumour was extensively spread in the breast, the ducts in the more peripheral parts of the tumour did not show any elastic hyperplasia while a section taken from the centre of the growth showed that it was present around some of the ducts in that area.

Discussion:

The sections examined were taken from the same tissue blocks used for the routine biopsy reports. About 80% of these sections showed an increase in the elastic tissue around the ducts in a mammary carcinoma.

The elastic hyperplasia occurred normally only in atrophic breasts, in women over 50 years of age, and involved all the main ducts. About one third of the 119 mammary carcinomata which showed elastic hyperplasia, occurred in women below the age of 50 years. The mammary carcinomata affecting women over this age were not present in atrophic breasts only. Furthermore, this elastic tissue increase was confined to the ducts in the centre of/

of the tumour area and was not present outside the tumour areas except in 8 out of the 119 cases. Cheatle and Cutler found a diffuse type of hyperplasia elastica around all the ducts and even most of the acini in a breast containing carcinoma. They do not give any figures about the prevalence of this condition.

All these findings led to the conclusion that the increased elastic tissue in breast carcinomata was not due to senile changes but was due to the presence of the tumour growth.

This new elastic tissue was produced by the host's tissues and not by the tumour. It was never found in any of the metastases of the same growth. It was only seen around ducts in the primary growths. When the tumour infiltrated rapidly, some few elastic fibres were found irregularly scattered about the growth. These were the remains of ducts and septa which were destroyed leaving their elastic tissue relatively intact since it is more resistant to tumour invasion.

It was suggested at one time that this elastic hyperplasia was part of the scirrhous reaction in some mammary carcinomata. The figures on p. 146 show that it was present in the encephaloid type of carcinomata as well. An analysis of the relation between the stroma reaction, the size of the tumour and the elastic hyperplasia showed that the latter was more frequent in the more scirrhous and slowly growing types of tumours.

The nature of the actual causative agent of this elastic proliferation is difficult to ascertain. It appears to cause a kind of local desmoplastic reaction on the host's tissues as a response to the early tumour growth. A/

A similar reaction was found in the elastica of the skin by McConnell, Bierich and others. As long as the neoplastic proliferation was inside the epidermis there was a local increase of the dermal elastic fibres. When the cells began to infiltrate the corium, the elastic fibres were destroyed. The mammary tissues (both epithelial and connective) have great proliferative powers (see Loeb, 1932), consequently the elastic tissue response is greater and more obvious than that present in the skin. When the malignant cells infiltrated through the surrounding tissues they also destroyed the elastic fibres (Figs. 104, 105, 106, 108, 109, 113 and 119).

The elastic hyperplasia in mammary carcinomata had the following characteristics:-

1. It was a local response to tumour growth and rarely occurred outside the primary growth.
2. Serial sections show that all the affected ducts were connected together. These ducts were present in the centre of the tumour.
3. Cheatle and Cutler found that secondary metastases of a mammary carcinoma in the other breast did not cause elastic hyperplasia there.
4. This elastic hyperplasia was present in carcinomata only. Section made of true primary mammary sarcomata (8 cases) did not show any elastic tissue proliferation around the ducts, but the reverse.

The/

The serial sections of the six blocks studied showed that a primary focus could be seen inside such ducts. The spread of the tumour throughout the ductal tree and through the elastic barriers could be better demonstrated than in ordinary sections.

The findings of Cheatle suggested also to him that the primary malignant process occurred inside the ducts and acini surrounded by the increased elastic fibres. In my series of cases this elastic hyperplasia was found only around such affected ducts.

It is unfortunate that a complete study of all the changes occurring in the breasts affected by carcinomata could not be carried out. The carcinomata giving negative findings of elastic tissue proliferation could not be investigated more fully to see whether a primary malignant focus surrounded by increased elastic tissue could be found. It can, however, be suggested that in breast carcinomata showing elastic hyperplasia around the ducts, the primary carcinomatous process originated from the duct epithelium (for more information about the origin of mammary carcinoma see Cheatle and Cutler, 1931, Dawson, 1932-1936, Kropassy, 1937, and Muir, J. of Path., 1941).

Another important finding is the elastic hyperplasia in the non-malignant diseased breasts. This elastic hyperplasia could not be explained as normal senile changes. We know of the possible origin of mammary carcinomata in hyperplastic cystic disease and duct papillomata (see Muir). It would be of great value if it could/

could be proved that the inception of malignant changes in these two conditions is accompanied or preceded by elastic hyperplasia around the mammary ducts. A careful histological investigation and a follow up of these cases would be of great value.

Concerning the elastic tissue formation, it appears that the malignant changes cause the elastic producing cells normally present in the walls of the ducts to proliferate and lay down new elastic fibres as a local reaction. It was not found around the acini as elastic tissue does not normally exist there. Later on, when the cancerous growth infiltrated through, they gradually disappeared.

Summary:

Elastic tissue hyperplasia occurs normally in senile atrophic mammary tissue. It occurs also pathologically in mammary carcinomata, as a local reactive response of the host's tissues to the tumour growth.

It is suggested that a further study of these elastic changes in relation to the site of mammary carcinoma origin and in relation to other suspected precancerous conditions, would be of academical and practical value to our knowledge of the pathogenesis and pathology of mammary carcinoma.

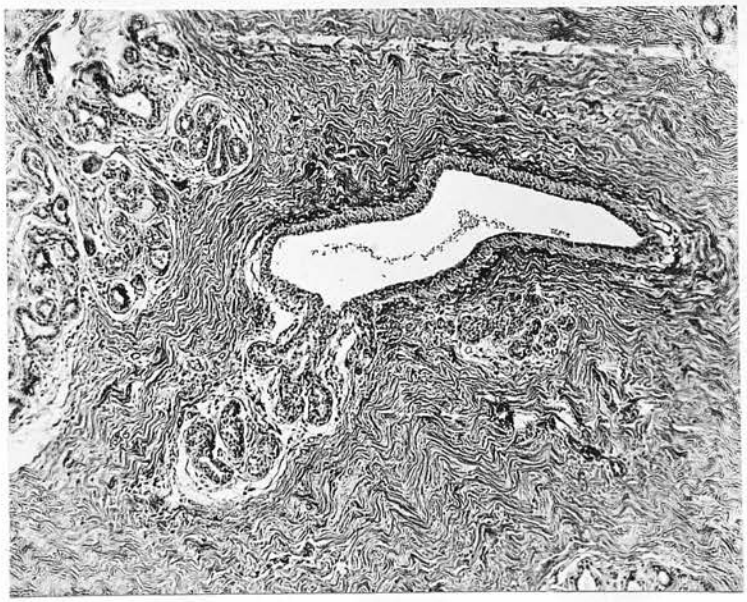

Fig. 94.

Mammary tissue from a woman, 34 years old. The elastic tissue surrounding the ducts does not extend to surround the acini but terminates abruptly where these begin.
Weigert - Haematoxylin - Van Gieson. x 75.

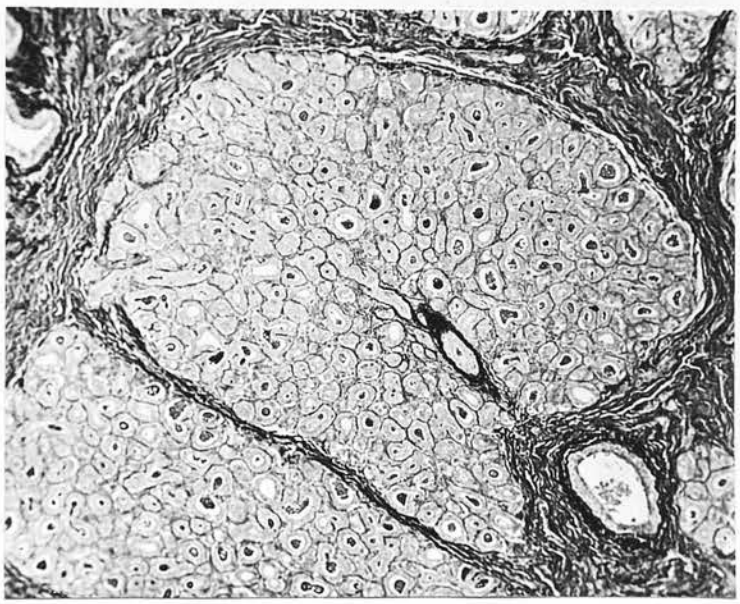

Fig. 95.

A mammary lobule from a woman 30 years old who died three days after delivery. Only the milk duct and its intralobular extension are surrounded by elastic fibres.
Weigert - Haematoxylin - Van Gieson. x 75.

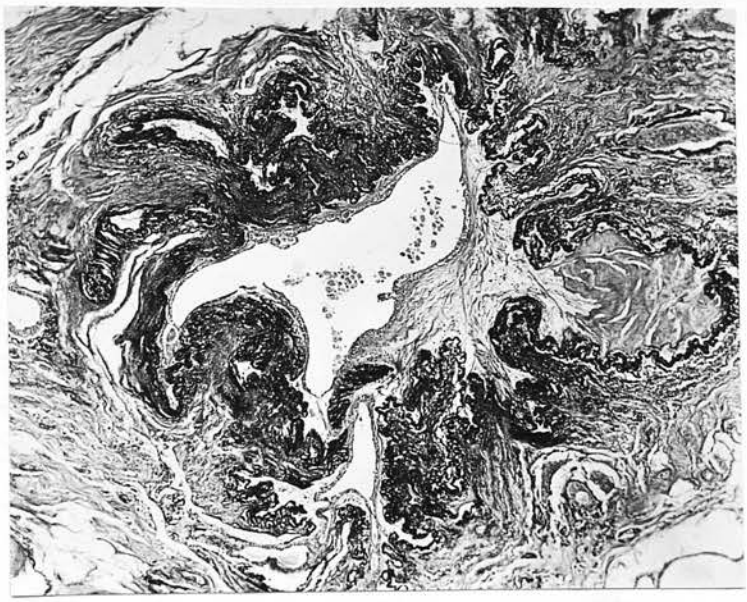

Fig. 96.

One of the remaining main milk ducts in a woman 74 years old. The breast was atrophied. Note the elastic hyperplasia and sub-epithelial fibrosis.
Weigert - Haematoxylin - Van Gieson. x 50.

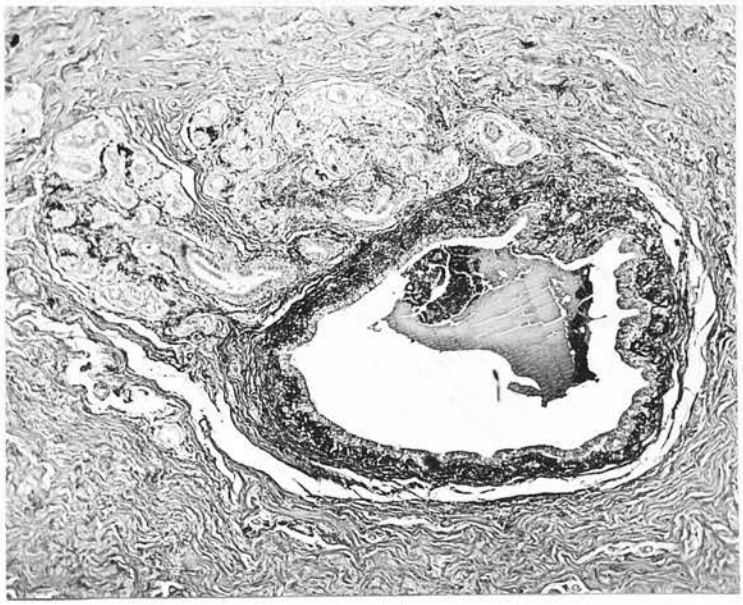

Fig. 97.

Hyperplastic cystic mastopathy in a woman 42 years old. One of the small mammary ducts is dilated and shows desquamative epithelial hyperplasia. There is an increased number of elastic fibres around it.
Weigert - Haematoxylin - Van Gieson. x 50.

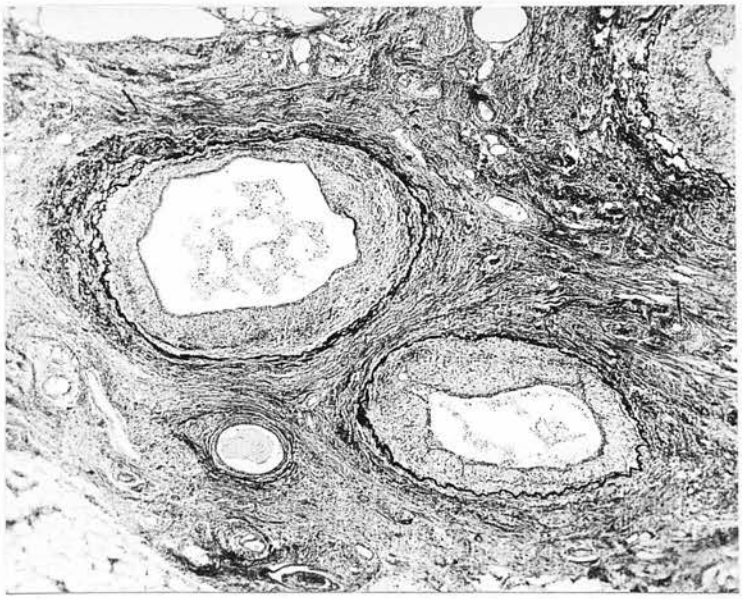

Fig. 98.

Hyperplastic cystic mastopathy in a woman 37 years old. The epithelium lining the dilated mammary ducts is normal but there is hyperplasia of both the sub-epithelial and elastic coats.

Weigert - Haematoxylin - Van Gieson. x 25.

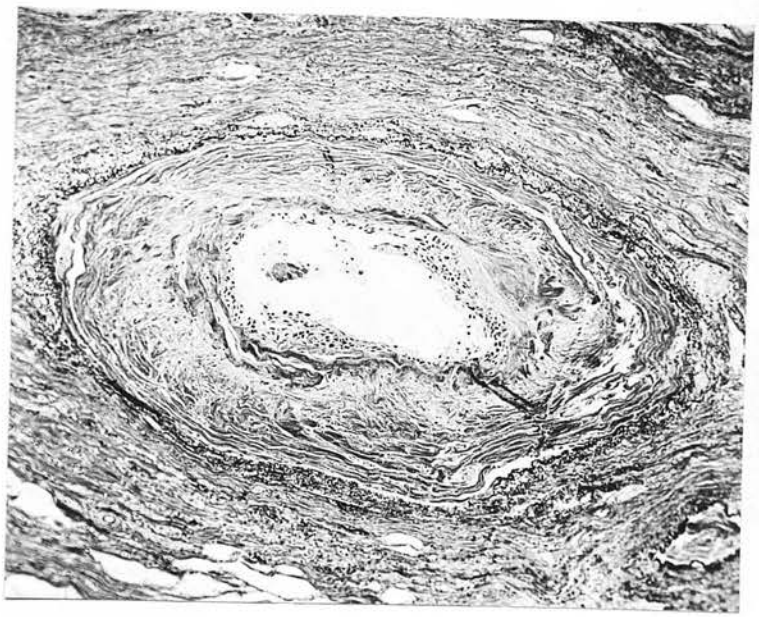

Fig. 99.

Hyperplastic cystic mastopathy in a woman 33 years old. There is a desquamative epithelial hyperplasia and increase in both the sub-epithelial and elastic coats.

Weigert - Haematoxylin - Van Gieson. x 75.

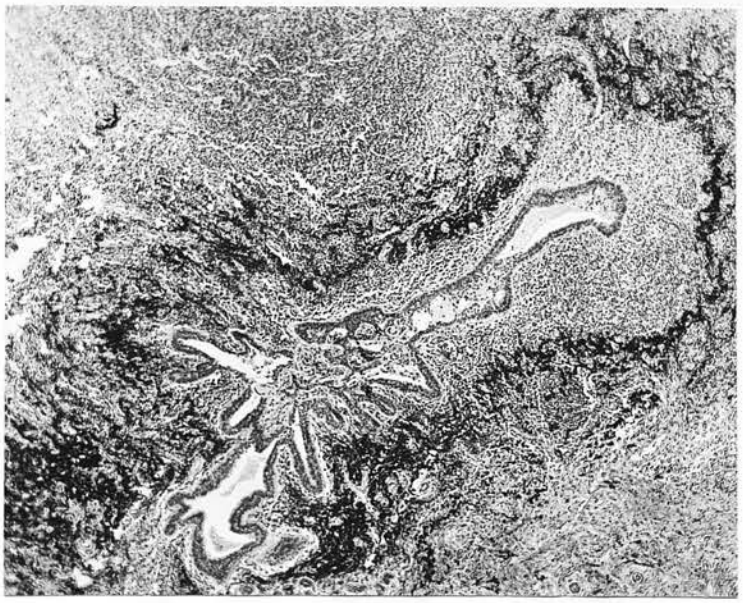

Fig. 100.

Subacute inflammatory mastitis in a pregnant woman, 32 years old. The epithelium lining the duct is normal. There is hyperplasia of both the sub-epithelial and elastic coats. The lumen and all the coats of the duct are infiltrated by inflammatory cells.

Weigert - Haematoxylin - Van Gieson. x 50.

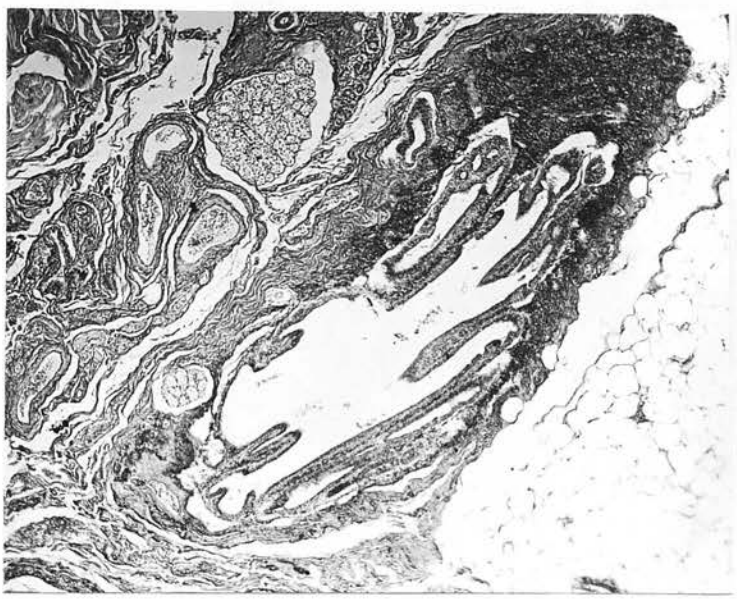

Fig. 101.

Mammary duct obstruction in a woman 33 years old. The cause was the development of duct papillomata. A small duct papilloma is shown in this picture. There is hyperplasia of the elastic tissue surrounding the duct.

Weigert - Haematoxylin - Van Gieson. x 60.

Fig. 102.

The whole of a scirrhous adeno-carcinoma (1 cm. in diameter) in the breast of a woman 68 years old. There is great elastic hyperplasia around the ducts in the centre of the tumour.

Weigert - Haematoxylin - Van Gieson. x 8.

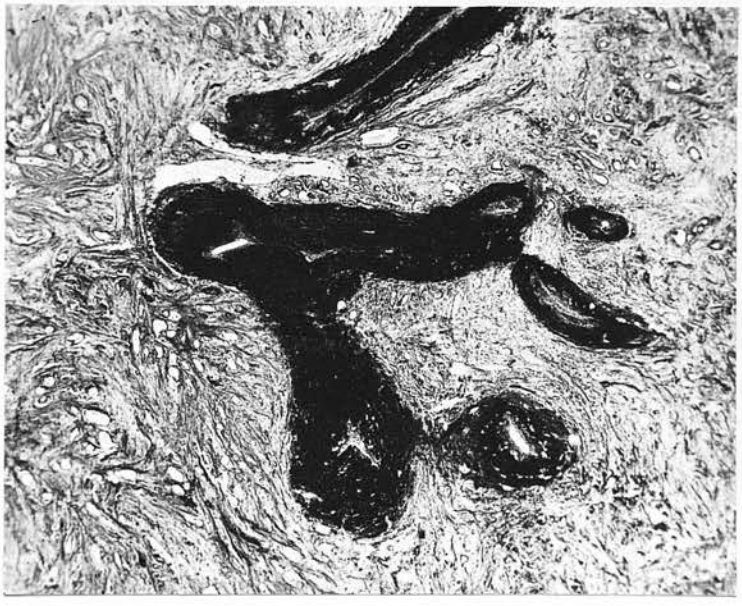

Fig. 103.

Scirrhous adeno-carcinoma (2 x 1 cm. diameter) in a woman 45 years old. The elastic hyperplasia around the ducts has practically occluded them. Outside the elastic layer the ducts are completely surrounded by malignant cells.

Weigert - Haematoxylin - Van Gieson. x 25.

Fig. 104.

Spheroidal cell scirrhous carcinoma (2 x 1½ cm.) in a woman 51 years old. The small ducts are surrounded by a thick coat of elastic tissue. The central duct is full of malignant cells which are spreading out through a break in the elastic coat. Note the granularity and destruction of the elastic tissue by direct contact with the malignant cells.
Weigert - Haematoxylin - Van Gieson. x 25.

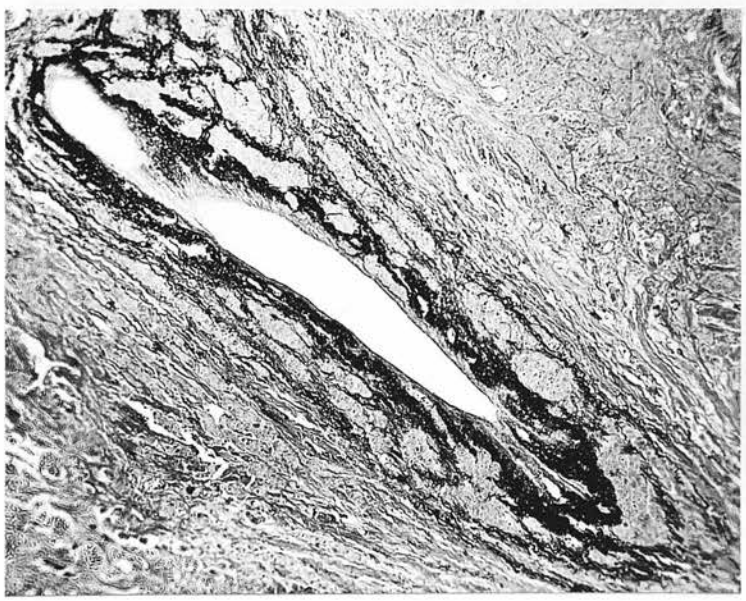

Fig. 105.

Anaplastic spheroidal cell carcinoma in a woman 48 years old. The lining of this duct is apparently normal. Cancer cells are infiltrating and destroying the hyperplastic elastic coat around the duct.
Weigert - Haematoxylin - Van Gieson. x 75.

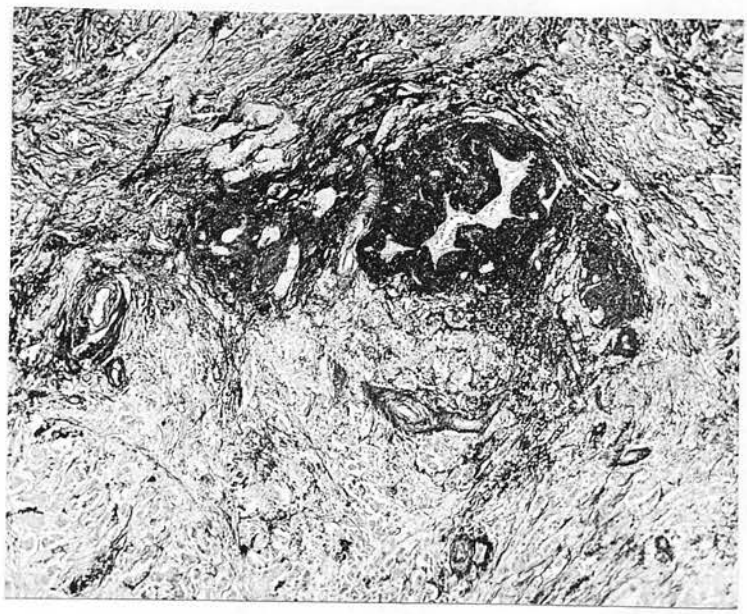

Fig. 106.

Spheroidal cell carcinoma (2 x 1.75 cm.) in a woman 49 years old. One of the small ducts is completely occluded, while in the other, the lining is apparently normal. Note the destruction of the elastic tissue by the malignant infiltration.
Weigert - Haematoxylin - Van Gieson. x 50.

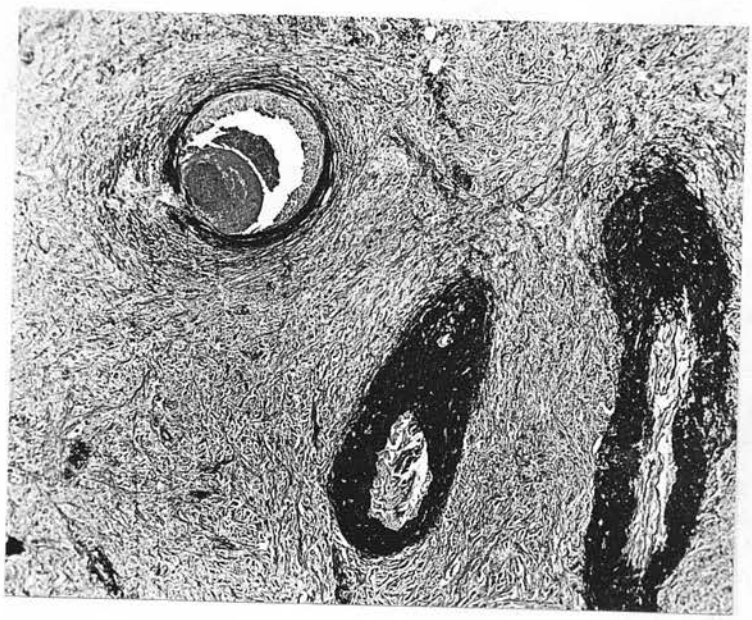

Fig. 107.

Scirrhous carcinoma in a woman 46 years old. Two of the ducts have their lumina filled with fibrous tissue. The third one is lined by malignant cells and shows a break in the elastic coat surrounding it.
Weigert - Haematoxylin - Van Gieson. x 25.

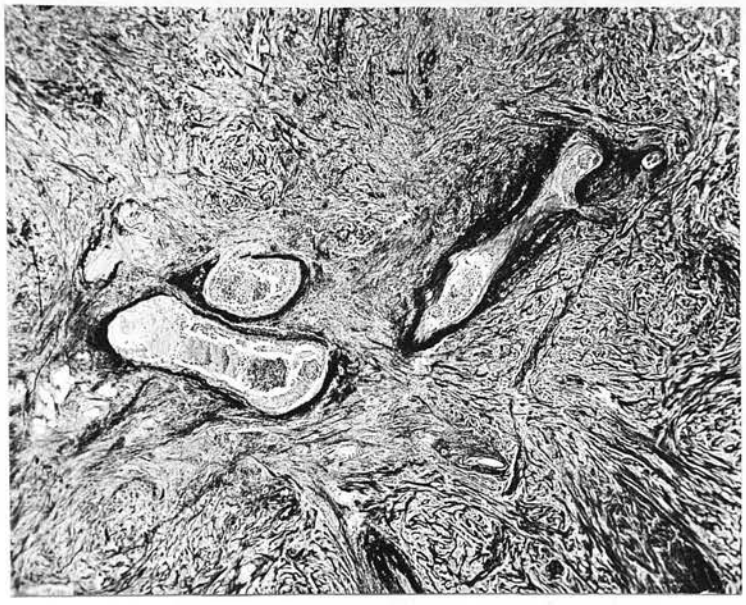

Fig. 108.

The central area of a mammary carcinoma (3 x 2½ cm.) in a woman 53 years old. The three ducts are filled with malignant cells, many of which are degenerated. There are several breaks in the elastic coats surrounding them.
Weigert - Haematoxylin - Van Gieson. x 25.

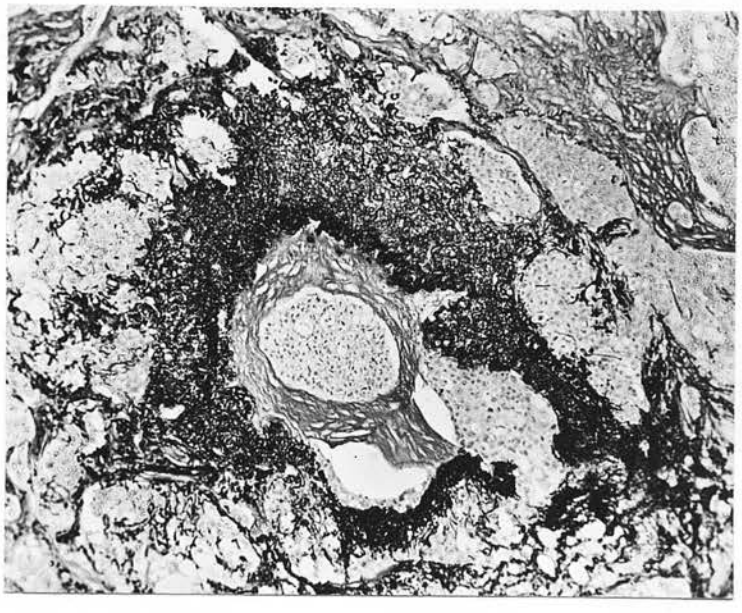

Fig. 109.

Scirrhous mammary carcinoma (5 x 2.5 cm.) in a woman 66 years old. The duct is full of malignant cells and shows both sub-epithelial and elastic hyperplasia. Note the breaking out of the malignant cells and the destruction of the elastic fibres.
Weigert - Haematoxylin - Van Gieson. x 75.

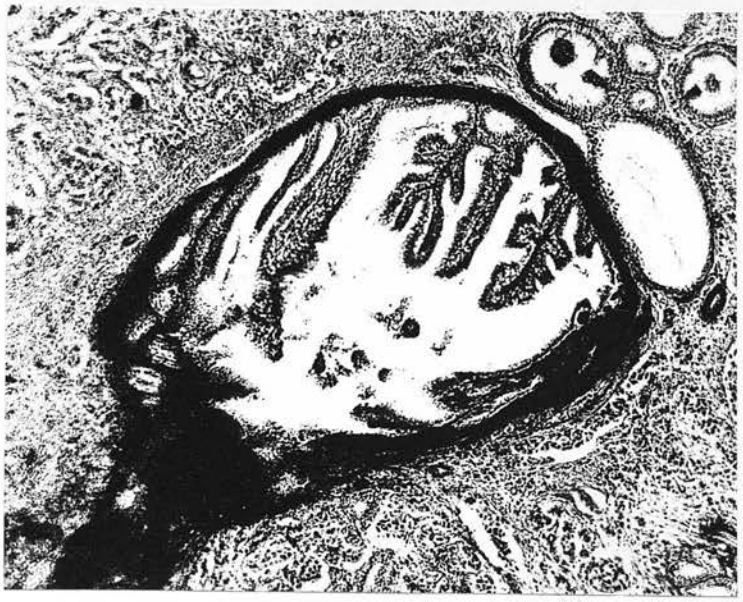

Fig. 110.

Mammary duct carcinoma ($4\frac{1}{2}$ x 2) in a woman 59 years old. It had infiltrated extensively with a marked scirrhous reaction. This picture shows a duct papilloma in the centre of the cancerous area. (Compare with Fig. 101.)
Weigert - Haematoxylin - Van Gieson. x 75.

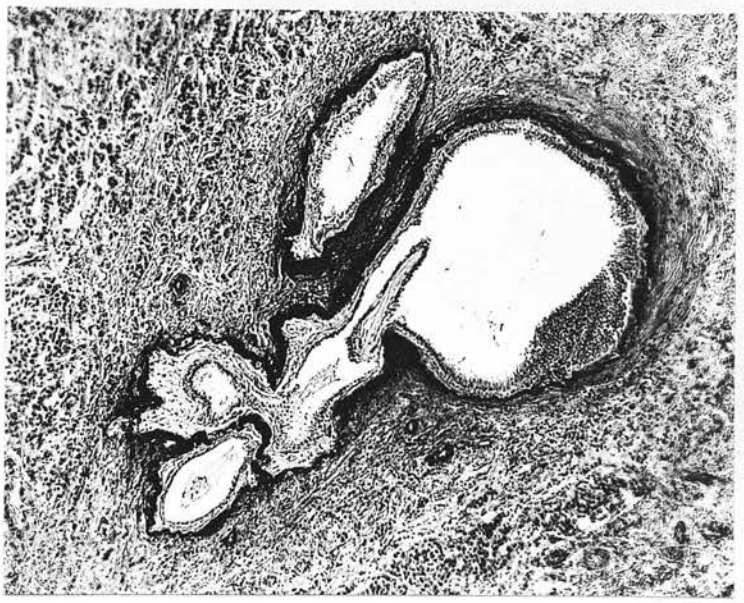

Fig. 111.

Same case as Fig. 110. The same duct further out is now lined by a slightly hyperplastic epithelium. Some parts show a mild sub-epithelial fibrous proliferation. (Compare with Fig. 97.)
Weigert - Haematoxylin - Van Gieson. x 75.

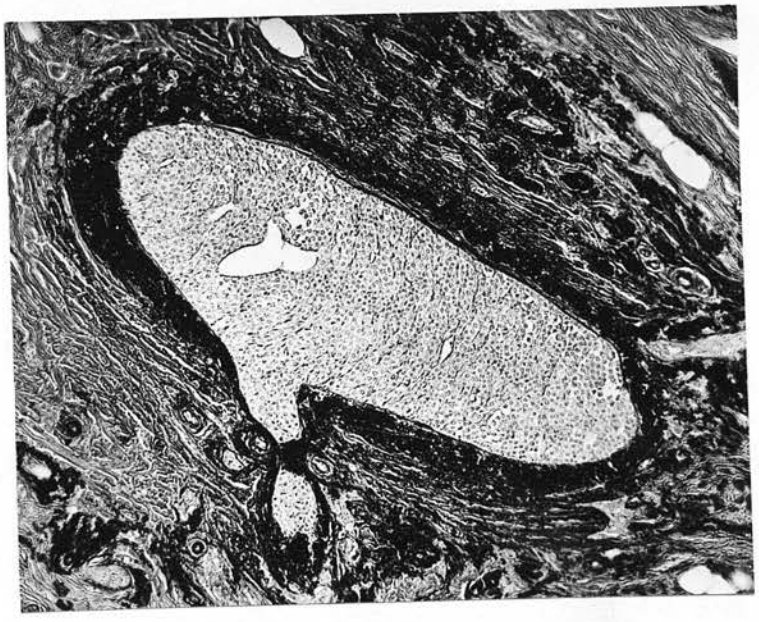

Fig. 112.

Same case as Fig. 110. A branch of the same ductal tree is now seen filled with malignant cells. At one side the cancer cells are breaking through the elastic coverings.
Weigert - Haematoxylin - Van Gieson. x 75.

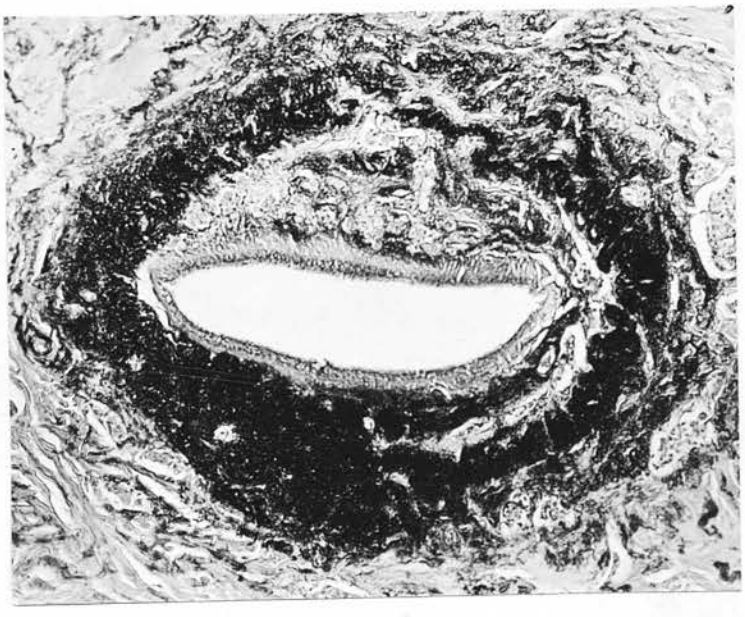

Fig. 113.

Same case as Fig. 110. A small duct continuous with that seen in Fig. 111 is seen. The lining epithelium is apparently normal. The sub-epithelial connective tissue is proliferated and invaded by malignant cells which are seen breaking through the elastic coverings.
Weigert - Haematoxylin - Van Gieson. x 110.

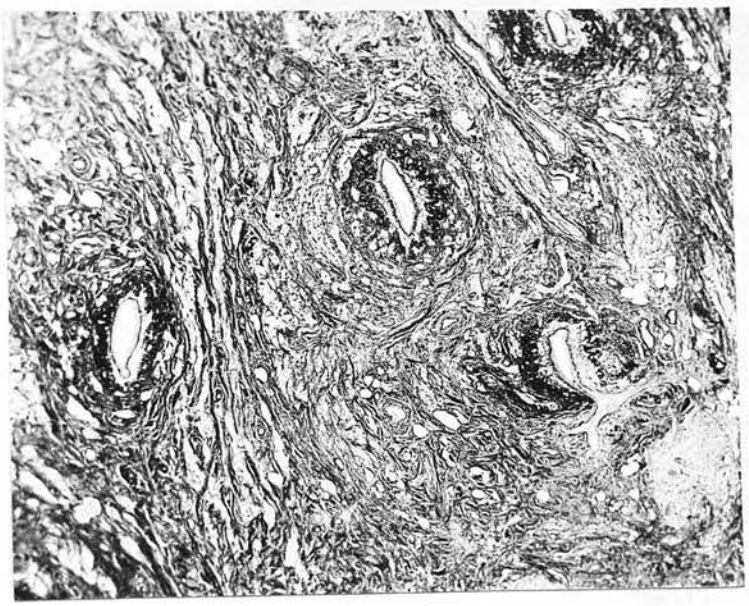

Fig. 114.

Same case as Fig. 110. This area was near the edge of the cancerous tissue. Some of the ducts have apparently normal epithelial linings. There is sub-epithelial fibrosis which is infiltrated by cancer cells. These are seen penetrating the elastic coats and spreading into the surrounding tissues.
Weigert - Haematoxylin - Van Gieson. x 25.

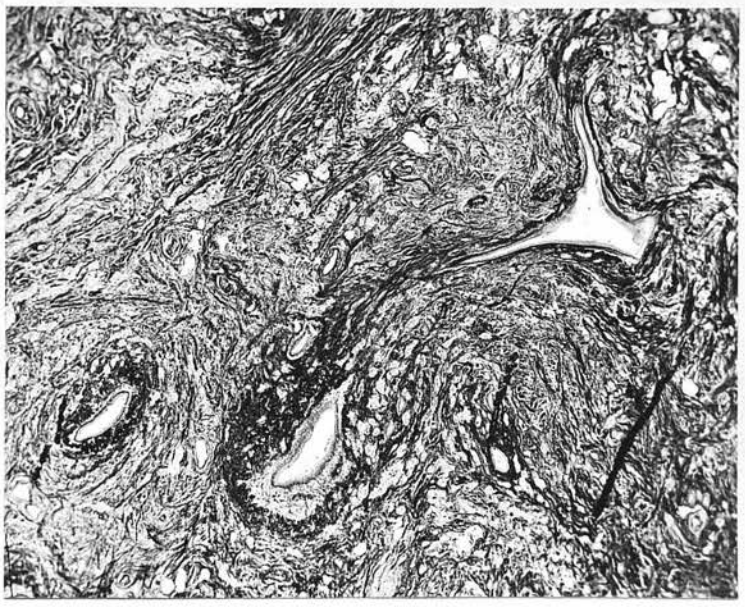

Fig. 115.

Another area from the same section shown in Fig. 114.
Weigert - Haematoxylin - Van Gieson. x 25.

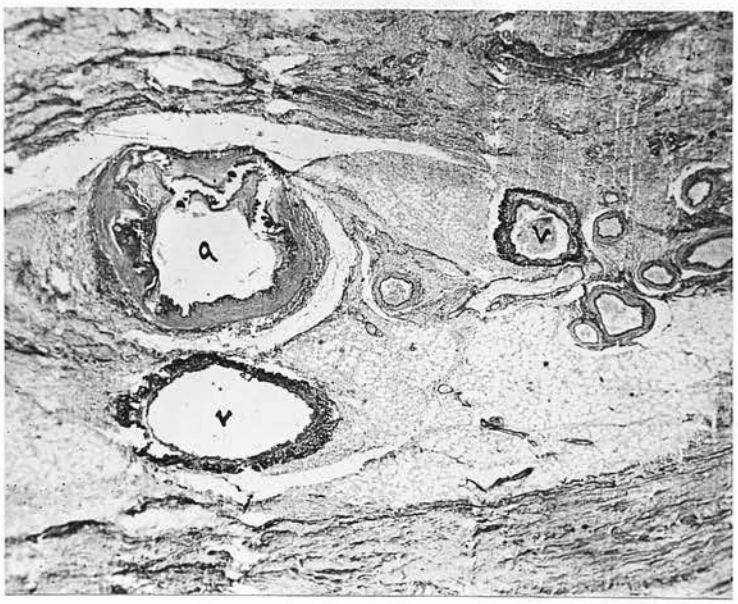

Fig. 116.

Mammary carcinoma in a woman 58 years old. The cancer cells have involved the tissues surrounding some of the blood vessels. The veins (v) show a marked elastic hyperplasia. The artery (a) shows calcification.
Weigert - Haematoxylin - Van Gieson. x 25.

Fig. 117.

Same case as shown in Fig. 103. One of the veins shows an extensive elastic hyperplasia. It is completely surrounded by malignant cells.
Weigert - Haematoxylin - Van Gieson. x 50.

ACKNOWLEDGMENTS.

I am deeply indebted to Professor A. Murray Drennan for his advice, guidance and encouragement throughout the work and for the generous hospitality of his Department; also to Dr. R. F. Ogilvie for his assistance and help with material from the Pathology Department, Royal Infirmary of Edinburgh; to Dr. A. C. P. Campbell for his valuable suggestions and helpful criticism; to Dr. G. M. Wilson for his co-operation in the arterial injection experiments. Dr. Mary K. MacDonald kindly read the original script and corrected many of the grammatical errors.

I should also like to acknowledge the help received from Dr. C. E. van Rooyen for his advice in the preparation of tissue cultures. Dr. A. W. Greenwood, of the Department of Animal Genetics, kindly supplied the chick embryos used in these experiments.

My thanks are due to Mr. T. C. Dodds and his Laboratory Staff for their assistance at all stages of the work. I specially express my appreciation for the care displayed by Mr. Dodds in the production of the photographic illustrations.

REFERENCES.

1. ALLEN, A. C. (1940): So-called Mixed Tumours of the Mammary Gland of dog and man.
Arch. Path. 29, 589.

2. ALLEN, E., BARKER, N. and HINES, E. (1946): Peripheral Vascular Disease.
Saunders, Philadelphia.

3. ALLEN, E. and CAMP, J. (1935): Arteriography.
J.A.M.A. 1904, 618.

4. AREY, L. B. (1934): Developmental Anatomy.
Saunders, Philadelphia.

5. ARGAUD, R. and DUCING, J. (1929): Hypergénes Elastique Intra-epitheliomateuse.
Ann. Anat-path. et Anat. Norm.Med-Chir.6, 37.

6. ASCHOFF, L. (1924): Lectures on Pathology.
New York.

7. ___ (1936): Pathologische Anatomie.
Fischer, Jena.

8. ASTBURY, W. T. (1939): X-ray Studies of Structures of Biological Interest.
Annual Rev. of Bio-Chem. 8, 113.

9. BAILY, O. T. (1934): Gastro-intestinal Argentaffinoma.
Arch. Path. 18, 843.

10. ___ (1940): Histologic Sequences in the Meningioma.
Arch. Path. 30, 42.

11. BAITSELL, G. A. (1915): The Origin and Structure of a Fibrous Tissue which appears in living cultures of adult frog tissue.
J. Exp. Med. 21, 455.

12. ___ (1916): The Origin and Structure of Fibrous Tissue formed in wound healing.
J. Exp. Med. 23, 739.

13. ___ (1917): A Study of the clotting of the Plasma of the frog's blood and the transformation of the clot into a fibrous tissue.
Am. J. Physiol. 44, 109.

14. ___ (1921): A Study of the development of connective tissue in Amphebia.
Am. J. Anat. 28, 447.

15./

15. BAKER, J. (1945): Cytological Technique.
Methuen, London.

16. BEATTIE and DICKSON (1943): Textbook of Pathology.
Heinemann, London.

17. BERKA, F. (1911): Die Brustdrüse Verschiedener Altersstufen und Während der Schwangerschaft.
Frankfurt. Zeit. F.Pathologie,8,203.

18. BIERICH, R. (1922): Uber die Beteiligung des Bindegewebes bei der Experimentellen Krebsbildung.
Virchow's Archiv. 239, 1.

19. BLACKWOOD, W. (1946): Observations on the Pathology of Peripheral Vascular Disease.
Postgraduate Med. J. 22, 1.

20. BLOOM, W. (1929): The Development of Elastic Fibres in Cultures of Embryonic Heart and Aorta.
Arch. Fur. Exp. Zellforsch, 9, 6.

21. ___ (1937): Cellular Differentiation and Tissue Culture.
Physiol. Rev. 17, 589.

22. ___ and SANDSTROM, R. H. (1935-36): The Development of Connective Tissue Fibres in Epithelial-containing Cultures.
Anat. Rec. 75, 64.

23. BRAMWELL, J. C. (1933): see Cowdry's Arteriosclerosis.

24. ___ McDOWELL and McSWINEY (1923): Vartiation of Arterial Elasticity with Blood Pressure in Man(Pt.1.)
Proc. Roy. Soc. Lond. 94, 450.

25. BORCHARDT, H. (1926): Endarterielle Gefässneubildung.
Virchow's Arch. 259, 372.

26. BORST, (1936): see Aschoff's Pathologische Anatomie.

27. BUERGER, L. (1924): The Circulatory Disturbances of the Extremities.
Saunders, Philadelphia.

28. ___ (1939): Thrombo-Angiitis Obliterans: Concepts of Pathogenesis and Pathology.
J.International de Chirurgie, 4, 399.

29. BUJARD, E. (1935): Quelque Donnés Experimentales sur la Reaction de coloration de fibres élastique.
Rev.Med. de la Suisse Romande,55,103.

30./

30. BUNTING, C. H. (1939): New Formation of Elastic Tissue in Adhesions between Serous Membranes and in Moyocardial Scars.
Arch. Path. 28, 306.

31. CAMERON, G. (1935): Essentials of Tissue Culture Technique.
Ferrar and Rinehart, New York.

32. CARLTON, H. M. and LEACH, E. H. (1938): Histological Technique.
Oxford Univ. Press, London.

33. CASTELMAN, B. (1940): Healed Pulmonary Infarcts.
Arch. Path. 30, 130.

34. CHEATLE, G. L. (1923): Hyperplasia of the Epithelium and Connective Tissue in the Breast.
Br. J. Surg. 10, 436.

35. ___ and CUTLER, M. (1931): Tumours of the Breast.
Arnold, & Co., London.

36. CLARK, E. R. (1936): The Growth and Development of Function in Blood Vessels and Lymphatics.
Ann. Int. Med. 9, 1043.

37. CONN, H. J. (1940): Biological Stains.
New York.

38. COWDRY, E. V. (1933): Arteriosclerosis. A Survey of the Problem.

39. ___ (1943): Microscopic Technique in Biology and Medicine.
London.

40. CRETIN and MATTET (1945): La Coloration des Fibres Elastique.
Bull. d'Hist. Appliq. 22, 107.

41. DAWSON, E. K. (1932): Sweat Gland Carcinoma of Breast.
Edin. Med. J. 39, 409.

42. ___ (1933): Carcinoma in the Mammary Lobule and its Origin.
Edin. Med. J. 40, 57.

43. ___ (1934): A Histological Study of the Normal Mamma in Relation to Tumour Growth (Pt. 1).
Edin. Med. J. 41, 653.

44. ___ (1935): do. (Pt. II).
Edinburgh Med. J. 42, 569.

45. ___ and TODD, M.C. (1934): Prognosis in Mammary Carcinoma.
Edin. Med. J. 41, 61.

46./

46. DAWSON, J. W. - see Beattie & Dickson: Textbook of Path., p. 53.

47. DAY, T. D. (1936): The Origin of Fibrous Connective Tissue in the Human Body.
J. Path. & Bact. 43, 49.

48. DOLJANSKI, L. and ROULET, F. R. (1933): Studien uber die Entstehung der Bindgewebsfibrille.
Virchow's Archiv. 291, 260.

49. DUBLIN, W. B. (1946): Reticulin.
Arch. Path. 41, 299.

50. DUGUID, J. B. (1946): Thrombosis as a Factor in the Pathogenesis of Coronary Atherosclerosis.
J. Path. & Bact. 58, 207.

51. DÜRCK, H. (1930): Die Sogenannte "Thromboangiitis Obliterans" in Rahmen der Infekiöstoxischen Gefässen-zundungen.
Verh. d. Deutsch. Path.Gesell.25,272.

52. EWING, J. (1940): Neoplastic Diseases.
Saunders, Philadelphia.

53. FAVRE, M. and MARTIN, J. F. (1937): Les Scléroses Elastigenes.
J. de Médecine de Lyon, 18, 679.

54. FISCHER, A. (1925): Tissue Culture Text Book.
Levin and Monksgaard, Copenhagen.

55. ___ (1946): Biology of Tissue Cells.
Cambridge Univ. Press.

56. ___ and PARKER, R. C. (1929): Proliferation und Differ-enzierung.
Arch. Exp. Zellforsch. 8, 297.

57. ___ and ___ (1929): Dauerzuchtung in Vitro Ohne Wachstumbeschleunigung.
Arch. Exp. Zellforsch. 8, 325.

58. FISCHER, B. (1904): Uber Neubildung von Elastin in Geschwulsten.
Virchow's Archiv. 176, 169.

59. FRENCH, R. W. (1940): A Combined Stain for Fat and Elastic Tissue.
Arch. Path. 30, 1243.

60. FULTON, J. S. and McSWINEY, B. A. (1930): Pulse-Wave Velocity and Extensibility of Brachial and Radial Artery in Man.
J. Physiol. 69, 386.

61./

61. FUSS, S. (1906): Die Bildung der Elastischen Faser.
Virchow's Archiv. 185, 1.

62. GEIPEL, P. (1906): Uber Elastischen Gewebe beim Embryo und in Geschwulsten.
Centralbl. f. Allg. Path u. Path. Anat. 17, 561.

63. GERY, L., FONTAINE, R. and BRANZEU, P. (1939): Les Lésion Chronique Obliterantes des Artéres des Membres.
J. International de Chir. 4, 427.

64. GOODALL, J. (1908-10): The Involution of the Puerperal Uterus.
Studies from the Royal Victoria Hospital, Montreal, Vol. II.

65. GRUBER, G. (1930): Endarteritis Obliterans und Kältebrand.
Ziegler's Beiträge, 84, 155.

66. HASS, G. (1939): a. Elastic Tissue.
Arch. Path. 27, 335.

67. ___ and MACDONALD, F. (1940): b. The Production of Collagen in Vitro.
Am. J. Path. 16, 525.

68. ___ (1940): c. Implantation of Collagen-forming Cultures in Granulation Tissue.
Am. J. Path. 16, 549.

69. ___ (1942): d. Methods of Isolation of Elastic Tissue.
Arch. Path. 34, 807.

70. ___ (1943): e. Elastic Tissue.
Arch. Path. 35, 29.

71. HATCHER, W. J. (1933): A Résumé of Elastic Staining Methods.
The Laboratory Journal, 35, 113.

72. HEUKING, E. and THOMA, R. (1887): Uber die Substitution des Marantischen Thrombus durch Bindgewebe.
Virchow's Archiv. 109, 288.

73. HINES, E. and BARKER, N. (1940): Arteriosclerosis Obliterans.
Am. J. Med. Sciences, 200, 717.

74. HORTON, B. (1930): A Study of the Vessels of the Extremities by Injection of Mercury.
S. Clin. North America, 10, 159.

75. HUECK, W. (1920): Uber das Mesenchyme.
Ziegler's Beiträge. 66, 330.

76./

76. HUXLEY, J. S. (1924): Early Embryonic Differentiation. Nature. 113, 276.

77. ___ (1932): Problems of Relative Growth.

78. JÄGER, E. (1932): Zur Pathologischen Anatomie der Thrombo-angiitis Obliterans bei Juveniler Extramitätengangrän.

79. JALOWY, B. (1937): Kollagen, Elastin und Reticulin der Haut . Zeit. f. Zellforsch. u. Mik. Anat.27,667.

80. JORDAN, H. E. (1939): Fibrillogenesis in Connective Tissue. Am. J. Anato. 65, 229.

81. JORDAN, LLOYD and SHORE (1938): The Chemistry of Proteins.

82. JORES, L. (1900): Zur Kenntnis der Regeneration und Neubildung des Elastischen Gewebes. Ziegler's Beiträge. 27, 381.

83. ___ (1907): Uber die Feinern Vorgänge bei der Bildung und Wiederbildung des Elastischen Bindgewebes. Ziegler's Beiträge. 41, 167.

84. KARRENSTEIN (1908): Ein Fall von Fibroelastomyxomen der Herzen. Virchow's Archiv. 194, 127.

85. KERR, J. G: (1919): Textbook of Embryology. Macmillan, London.

86. KERVILY, M.de (1924): Compt. Rendu de la Soc. de Biol., 90:
a. Structure Granuleuse des fibres Elastique Révélée par L'impregnation a L'argent (p.736).
b. Les Fibrilles Elastique dans les Lames Collagéne (p. 581).
c. Les Granulation des Elastoblastes et les premier Stades de Développement des Fibres Elastique Révéles par L'impregnation at L' argent (p. 1022).
d. A propos des Granulation des Fibres Elastiques. (p. 1457).

87. KING, J. (1930): Tissue Culture Technique. Arch. Exp. Zellforsch. 9, 341.

88. KING, L. S. (1938): Vital Staining of Connective Tissue. J. Exp. Med. 68, 63.

89. ___ (1938): Normal and Pathologic Factors in Spreading Dyes in Connective Tissue Fibres. J. Exp. Med. 68, 869.

90. KISS, F. (1921): Anatomisch-Histologische Untersuchungen Über die Erektion. Zeitschr. Anat. u. Entw. 61, 455.

91./

91. KRAJIAN, A. A. (1934): A new Elastic Tissue Stain.
Arch. Path. 18, 378.

92. KRAUPSE, C. (1922): Beiträge zur Kenntnis der Gitterfasern.
Virchow's Archiv. 237, 474.

93. KROMPECHER, S. (1928): Die Entwicklung der Elastischen Elemente der Arterienwand.
Zeit. f. Anat. u. Entwick, 85, 704.

94. ___ (1930): Teleangiostenose, Arteriosclerosis Renum und Scleroderma.
Ziegler's Beiträge. 85, 647.

95. KROPASSY, B. (1937): Systematische Untersuchungen uber Epithelveranderungen in der Weiblichen Brustdrüse.
Virchow's Archiv. 299, 793.

96. KRZYSZTALOWICZ, F. (1903): see Encyklopädie der Mikroskopischen Tecknik.
Berlin.

97. LANGERON, N. (1934): Précis de Microscopie.
Paris.

98. LAWSON, W. H. (1934): Elastic Fibres in Sputum.
J. Path. & Bact. 39, 703.

99. ___ (1936): Modification of Weigert-Sheridon Stain.
J. Tech. Methods. 16, 42.

100. LEARY, T. (1934): Atherosclerosis.
Arch. Path. 17, 453.

101. ___ (1941): Genesis of Atherosclerosis.
Arch. Path. 32, 507.

102. LEE, A. B. (1937): The Microtomist's Vade Mecum.

103. LENDRUM, A. C. (1935): Celestine Blue as a Nuclear Stain.
J. Path. & Bact. 40, 415.

104. ___ (1946): Elastic Stains (Personal Communication).

105. ___ (1947): See Recent Advances in Clinical Pathology.

106. LERICHE, R. (1945): Thromboses Artérielles.
Paris, 1946.

107./

107. LEWIS, D. and REICHER, F. (1926): The Collateral Circulation in Thrombo-angiitis obliterans. J.A.M.A. 87, 302.

108. LEWIS, M. R. (1917): Development of Connective Tissue Fibres in Tissue Cultures of Chick Embryos. Contr. Embryol. Carnegie Instit.6,226.

109. LOEB, L. (1932): The Cytology of the Mammary Gland in Cowdry's special Cytology, p. 1633.

110. LOISEL, G. (1897): Formation et évolution des élément du tissu elastique. J. Anat. et physiol. 33, 129.

111. LOWRY, O. H., GILLIGAN, D. R. and KATERSKY, E. M. (1941): The Determination of Collagen and Elastin in Tissues. J. Biol. Chem. 139, 795.

112. McCONELL, G. (1907): The Elastic Tissue of Carcinomata. J. Med. Research. 16, 7.

113. McKINNEY, R. L. (1930): The Development of Reticulin into Collagen Fibres in Cultures of Adult Rabbit Lymph Nodes. Arch. F. Exp. Zellforsch, 9, 14.

114. McLUNG, C. E. (1929): Handbook of Microscopical Technique. New York.

115. MALL, F. P. (1901): The Development of Connective Tissues from Tissue Syncytium. Am. J. Anat. 1, 331.

116. MALLORY, F. B. (1938): Pathological Technique. London.

117. MANN, G. (1902): Physiological Histology. Oxford.

118. MAXIMOW, A. (1925): Tissue Culture of Young Mammalian Embryos. Contrib. to Embryol. Carnegie Inst.16,47.

119. ___ (1928): Development of Argyrophil and Collagenous Fibres in Tissue Culture. Proc.Soc.Exp.Biol. & Med. 25,439.

120. ___ and BLOOM, W. (1942): Textbook of Histology. Saunders.

121./

121. MILNE, L. (1908): Thesis for the degree of M. D. University of Edinburgh.

122. NAGEOTTE, J. and GUYON, L. (1930): Reticulin. Am. J. Path. 6, 631.

123. NAKAI, M. (1905): Uber die Entwicklung der Elastische Fasern in Organismus und ihre Beziehungen z. d. Gewebsfunkion. Virchow's Archiv. 182, 153.

124. ODIETTE, D. (1934): La Fibre Elastique dans les Culture des Tissue in Vitro. J. de Physiol. et de path. Generale.32,715.

125. ORSÓS, F. (1926): Das Bindgewebsgerüst der Lymphknoten im Normalen und Pathologischen Zustand. Ziegler's Beiträge. 75, 16.

126. PARKER, R. C. (1933); The Races that constitute the group of Common Fibroblasts. J. Exper. Med. 58, p. 97-113 and 401-414.

127. ___ (1936): The Cultivation of Tissues for prolonged periods in Single Flasks. J. Exper. Med. 64, 121.

128. ___ (1938): Methods of Tissue Culture. Hoeber, New York.

129. PATERSON, J. C. (1936): Vascularisation and Haemorrhage of the Intima of Arteriosclerotic Coronary Arteries. Arch. Path. 22, 313.

130. PATTEN, B. M. (1925): Early Embryology of the Chick. London.

131. PETRUNKEVITCH, A. (1937): On Differential Staining. Anat. Rec. 68, 267.

132. POPKEN, C. (1936): Uber Juvenile Spontangangrän. Ziegler's Beiträge. 97, 396.

133. PORTA, E. (1930): Differenziazione di Fibre Elastiche in Colture in Vitro. Bull.d.Soc.Ital.di Biol.Sper. 5, 327.

134. RAMSEY, E. M. (1936): Nutrition of the Blood Vessel Wall. Yale J. of Biol. & Med. 9, 14.

135./

135. REIDEL, G. (1925): Die Entwicklung u. Entartung des Elastischen Gewebes in der Senilen Mamma. Virchow's Archiv. 256, 243.

136. RINEHART, J. F. (1930): Reticulum. Am. J. Path. 6, 525.

137. ROMEIS, B. (1932): Taschenbuch der Mikro-Technik. Munich.

138. RÖTHIG, P. (1907): Entwickelung der Elastischen Fasern. Ergeb. der Anat. u. Entwick, 17,300.

139. SAVIN, E. and SAVINI-CASTANO, T. (1909): Uber das Elastische Gewebe der Mamilla in Normalen und Pathologischen Zustande. Virchow's Archiv. 198, 459.

140. SAXTON, J. A. (1942): Elastic Properties of Rabbit Aorta in Relation to Age. Arch. Path. 34, 262.

141. SCHEEL, O. (1906): Uber Neubildung des Elastische Gewebes in Karcinomen Besonders der Mamma. Ziegler's Beiträge. 39, 187.

142. SCHILLING, F. (1925): Experimentelle Erzeugung von Intimahyperplasien. Verh.d.Deutsch. Path. Ges. 20, 154.

143. SCHLESINGER, M. J. (1938): Injection plus dissection study of Coronary Artery Occlusions and Anastomoses. Am. Heart J. 15, 529.

144. SCHMORL, G. (1914): Die Pathologische-Histologischen Untersuchungsmethoden. Leipzig.

145. SEKIGUCHI, S. (1917): Studies on Paget's Disease of the Nipple. Annals of Surg. 65, 175.

146. SPONHEIMER, K. (1929): Zur Frage der Anatomischen Grundlegen der Spontängangrän. Ziegler's Beiträge. 82, 122.

147. STEARNS, M. L. (1940): Studies on the Development of Connective Tissue in transparent chambers in the rabbit's ear. Am. j. Anat. 67, 55.

148. STEIN, W. H. and MILLER, E. G. (1938): The Composition of Elastin. J. Biol. Chem. 125, 599.

149./

149. STRANGEWAYS, T. S. P. (1924): The Technique of Tissue Culture "in Vitro."
Heffer & Sons, Cambridge.

150. TANNENBERG, J. and FISCHER-WASELS, B. (1927): Die Sekundaren Veranderungen des Thrombus.
P. 1735 in Handbuch der Normalen und Pathologische Physiologie, VII, 2.

151. THURINGER, J. M. (1925): A new Method for staining Elastic Fibres.
J. Lab. and Clin. Med.89, 11.

152. TICHOMIROV, D. M. (1934): Uber die Wirkung der Fermente Maligner Geschwulste auf Elastische Gewebe in Vitro.
Virchow's Archiv. 292, 310.

153. TRITCHOWITCH, Y. (1922): L'action de l'autolyse sur le tissue elastique.
Compt. Rend. Soc. Biol. 87, 1135.

154. UNNA, P. G. (1896): Histopathology of the Skin.
New York.

155. ___ (1928): Histochemie der Haut.
Berlin.

156. VAN ROOYEN, C. E. and RHODES, A. J. (1940): Virus Diseases of Man. (Chapter IX).
Oxford.

157. WALJASCHKO, G. A. (1907): Uber das Elastische Gewebe in Neubildungen.
Virchow's Archiv. 187, 286.

158. WALLART, J. and HOUETTE, C. (1934): Une Coloration trichromique rapide.
Bull. d'Hist. Appl. 11, 404.

159. WILENS, S. (1937): The Post Mortem Elasticity of the Adult Aorta.
Am. J. Path. 13, 811.

160. WILLIS, R. A. (1934): Spread of Tumours in the Human Body.
London.

161. WILLMER, E. N. (1935): Tissue Culture.
Methuen, London.

162. WINTERNITZ, M. C., THOMAS, R. M. and LE COMPTE, P. M. (1938): The Biology of Arteriosclerosis.

163. WOLF, J. (1931): See Biological Abstracts, 1931, Abst. no. 18566.

164. YOUNG, J. Z. (1945): The Structure, Degeneration and Repair of Nerve Fibres.
Nature, 156, 132.